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The incidence of obesity is increasing worldwide, currently affecting over 400 million people. With obesity, expansion of white adipose tissue (WAT) contributes to a chronic, low-grade inflammatory response that is associated with the formation of complications such as type 2 diabetes, cardiovascular disease, and hypertension. One potential method of reducing chronic inflammation associated with obesity is through consumption of table grapes, which contain fiber and are rich in phytochemicals with potential health promoting properties. Several clinical trials and animal studies have demonstrated that consumption of grape products (i.e. grape seed extract, grape juice, or California powdered table grapes) can reduce oxidative stress, insulin resistance, and inflammation, as well as improve heart health. However, there is still much to be elucidated concerning the mechanisms by which grapes or grape products exert beneficial effects and which compounds within grapes are active in the mediation of these effects. Additionally, the influence of grape polyphenols and fiber on gut microbiota and the link of this with improvements in adiposity, systemic inflammation and insulin resistance are unclear.

Therefore, the specific aims of this research were to (i) determine the extent to which California table grapes attenuate body fat accumulation, systemic inflammation, and insulin resistance, and impact gut microbiota in mice fed and American type diet rich in

butter (Aim 1), and (ii) identify the key bioactive fraction(s) responsible for reducing adiposity, inflammation, and insulin resistance, and modulating gut microbiota in mice fed an American-type diet rich in four types of saturated fat (Aim 2). In Aim 1, grape consumption at one or both levels (i.e. 3% or 5% w/w) attenuated accumulation of body and liver fat, but these lipid lowering effects were not associated in improvements in glucose tolerance or markers of inflammation in WAT. Alterations in microbial species (i.e. reductions in deleterious sulfidogenic bacteria and increases in beneficial bacteria) were observed in grape-fed mice. Taken together these data demonstrate that consuming grapes results in attenuations in adiposity and hepatic steatosis, and also alterations gut microbial populations in mice fed a butter-rich diet. In Aim 2, consumption of the extractable polyphenol fraction (EP) alone or with the non-extractable polyphenol fraction (EP+NEP), but not powdered grapes (GP), reduced adiposity, liver and plasma triglycerides, markers of inflammation within WAT, and improved insulin sensitivity in mice fed a diet rich in saturated fat from four sources. Taken together, these data demonstrate that (i) consumption of polyphenols extracted from powdered grapes is effective at preventing the complications of diet-induced obesity, and that (ii) the effects of powdered grapes differ based on the amount and source of dietary fat. Overall, the findings from Aim 1 and Aim 2 are anticipated to contribute to the development of novel dietary strategies using grapes or grape products to manage or treat diet-induced obesity and associated conditions. Further research studies, including clinical trials, are still warranted to determine the applicability of these findings to the human population.

IMPACT OF CALIFORNIA TABLE GRAPES ON SYSTEMIC INFLAMMATION, INSULIN RESISTANCE, AND HEPATIC STEATOSIS IN MICE FED AN AMERICAN-TYPE DIET

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Overview

Obesity is a global health condition with approximately 400 million people classified as obese and 1.6 billion overweight [1]. The sharp rise in obesity is paralleled by similar increases in the incidence of cardiovascular disease (CVD), hypertension, and type 2 diabetes, all of which are associated with chronic, low-grade inflammation [2]. These obesity-related conditions are referred to as the metabolic syndrome. Primary causes of obesity are overconsumption of diets rich in fat, refined carbohydrates, and sugars, and lack of physical activity.

One potential way to reduce chronic inflammation associated with diet-induced obesity is to consume table grapes. Grapes contain fiber and phytochemicals with potential health-promoting properties [reviewed in 3]. For example, several clinical trials have demonstrated that consuming grape seed extract, grape juice, or California powdered table grapes decreased oxidative stress and inflammatory markers within blood [4, 5, 6]. It has also been demonstrated that consumption of grape seed extract decreased colon cancer in mice [7]. Additionally, our lab group has shown that California table grape supplementation decreased insulin resistance acutely, and systemic inflammation chronically, but not adiposity in mice fed an extremely high fat, lard-based diet [8].

However, the extent to which California table grapes reduce adiposity, systemic inflammation, and insulin resistance in mice fed an American type diet rich in saturated fat, and their mechanism of action including their potential prebiotic actions, have not yet been reported.

Central Hypothesis and Specific Aims

This thesis is a part of a continuing project with the *long-term goal* of developing novel approaches to prevent or treat obesity and its metabolic consequences using table grapes. The *central hypothesis* of my thesis research was that California table grapes, and one or more of their bioactive fractions or components, would decrease adiposity, systemic inflammation, and insulin resistance associated with diet-induced obesity. I predicted these beneficial systemic outcomes would be due, in part, to modulation of the gut microbiota and subsequent improvements in intestinal health. The working model showing how grapes exert these effects is shown in Figure 1.1.

To test my central hypothesis, the following *specific aims* were pursued:

Aim #1. Determine the extent to which California table grapes attenuate body fat accumulation, systemic inflammation, and insulin resistance, as well as impact gut microbiota in mice fed an American type diet rich in saturated fat from butter; and

Aim #2. Identify the key bioactive fractions in California table grapes responsible for reducing adiposity, inflammation, and insulin resistance, and modulating gut microbiota in mice fed an American type diet rich in saturated fat from four sources.

The *expected outcomes* of these aims were as follows:

Aim #1. I expected that whole powdered grapes would reduce adiposity, systemic markers of inflammation, and insulin resistance, which would be due, in part, to its pre- or postbiotic actions.

Aim #2. I expected that the methanol-extractable, polyphenol-rich fraction of powdered grapes would elicit greater improvements in adiposity, inflammation, and insulin resistance compared to the non- extractable fraction due, in part, to beneficial alterations in gut microbiota and improvements in intestinal health.

This thesis was innovative and significant, because it was expected to identify novel mechanisms by which whole California table grapes alter gut microbiota and intestinal health that, in turn, cause reductions in systemic inflammation and insulin resistance. Such knowledge would provide great benefit to the medical community and the public by informing them about the pre- and postbiotic properties of California table grapes, the most abundant source of table grapes in the U.S.

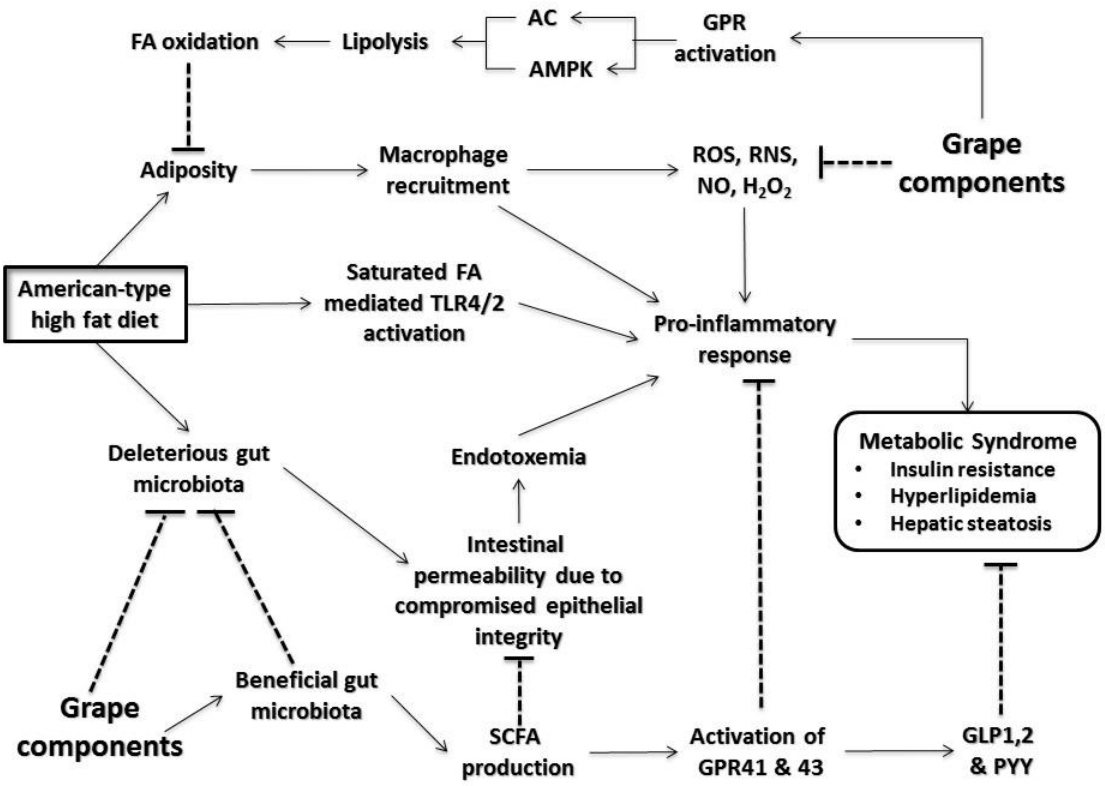

Figure 1.1. Proposed Model on how Table Grapes Attenuate the Development of Diet-Induced Obesity, Inflammatory Signaling, and Insulin Resistance. As prebiotics, grape polyphenols enhance the abundance of healthy types of bacteria (e.g, *Akkermansia muciniphila*) that contribute to short chain fatty acid (SCFA) production and activation of G-protein receptors 41 and 43 (GPR41 and GPR43) resulting in suppression of complications associated with consuming a high fat diet (i.e. inflammation and metabolic syndrome). Grape polyphenols also decrease the abundance of noxious bacteria (e.g., sulfidogenic bacteria) that impair gut barrier functions (i.e., cause leaky gut). These improvements in barrier function prevent endotoxemia (i.e., increase LPS in the bloodstream)-mediated inflammatory gene expression systemically. Alternatively, grape polyphenols reaching the blood stream may directly attenuate saturated FA mediated nutritional toxemia (e.g., saturated FA mediated TLR4/2 activation) that triggers white adipose tissue inflammation leading to metabolic syndrome. Grape

polyphenols may also activate GPRs thereby activating adelylate cyclase (AC) and 5’ adenosine monophosphate kinase (AMPK) which increase lipolysis and fatty acid (FA) oxidation in turn attenuating adiposity, subsequent macrophage recruitment and reactive oxidant species (ROS), reactive nitrogen species (RNS), nitric oxide (NO), and hydrogen peroxide (H2O2) production associated with high fat diet consumption.

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CHAPTER II

REVIEW OF LITERATURE

Obesity Epidemic and White Adipose Tissue (WAT)

Over the last few decades the incidence of obesity has increased drastically throughout the world. Recent estimates indicate that there are greater than 1.45 billion overweight individuals worldwide, with approximately 500 million of these individuals being obese [1]. This rise in obesity has resulted in large increases in related health conditions such as type 2 diabetes, hypertension, hyperlipidemia, cardiovascular disease (CVD), and fatty liver disease [2]. Collectively, this group of obesity-related diseases has been referred to as the metabolic syndrome. Notably, the metabolic syndrome is intimately linked with chronic inflammation in WAT that contributes to systemic inflammation and insulin resistance [1].

Relationship of Obesity and WAT Expansion to Inflammation

Increased adiposity is associated with a positive energy balance, resulting in adipocyte hyperplasia and hypertrophy. Adipocyte hypertrophy, in turn, activates oxidative stress and inflammatory signaling pathways [reviewed in 3]. Up-regulation of these pathways, in turn, impairs glucose and fatty acid uptake and lipid synthesis via inhibition of insulin signaling. This inflammatory response has been linked to several specific pathways. In

adipocytes, in vitro and in vivo evidence indicates that oversupply of lipid results in up-regulation of toll-like receptors (TLR2/TLR4), which in turn is associated with activation of stress-associated kinases such as c-Jun N-terminal kinase (JNK), mitogen activate protein kinase (MAPK) p38, extracellular signal-regulated kinase (ERK), protein kinase C (PKC), nuclear factor kappa B (NFκB), and activator protein (AP)-1 (aka cJun) resulting in transcription and translation of downstream proinflammatory peptides and proteins [4]. For example, TLR-4 deficient mice are protected against the obesity related pro-inflammatory effects of a high fat diet. Furthermore, TLR-4 deficient mice had reduced levels of cellular NFκB and circulating monocyte chemoattractant protein (MCP)-1 levels [5], demonstrating a linkage between fat-induced TLR activation and inflammation. Obesity has also been shown to activate the inflammasome, a cytosolic multi-protein complex in white adipose tissue (WAT), leading to insulin resistance and an increase in the inflammatory cytokine interleukin (IL)-1β [6]. It has also been shown that feeding C57BL/6J mice a high fat diet for 16 weeks increased the expression of genes related to inflammation within epididymal WAT, resulting in WAT apoptosis and remodeling [7].

Effect of Polyphenols on Inflammation and Obesity

Polyphenols are naturally occurring phytochemicals found in plants. They exhibit a wide range of biological activities, particularly suppressing inflammation and oxidative stress. Polyphenols have been shown to act as antioxidants, reducing the levels of reactive oxygen species (ROS), reactive nitrogen species (RNS), hydrogen peroxide

(H2O2), and nitric oxide (NO) which in turn blocks the pro-inflammatory pathways within WAT including the NFκB, activator protein 1 (AP-1), and mitogen-activated protein kinase (MAPK) pathways [reviewed in 8]. Additionally, polyphenols may inhibit pro-oxidant associated oxidative damage and cell death through activation of nuclear factor erythroid 2 related factor 2 (Nfr2) leading to increases in expression of numerous antioxidant enzymes [9]. Polyphenols have also been shown to reduce insulin resistance, most notably due to the ability of attenuate inflammatory or pro-oxidant signals that impair insulin-stimulated glucose uptake. Anti-inflammatory and anti-diabetic effects have been demonstrated for a wide range of polyphenols including curcumin from turmeric, epigallocatechin gallate (EGCG) from green tea, and resveratrol from wine, grapes, and berries [reviewed in 8]. Additionally, polyphenols have been shown to suppress lipogenesis and increase beta-oxidation via cAMP, AMPK, and SIRT1 pathways, resulting in increased glucose and fatty acid oxidation [reviewed in 8, 10]. The anti-inflammatory and anti-oxidant properties of polyphenols are depicted in Figure 2.1.

While polyphenols have been observed to have many anti-oxidant and inflammatory actions systemically, and some studies indicate that these compounds may aid in the prevention or reduction of obesity, there is still much to be elucidated. Many of the studies mentioned use individual polyphenols at pharmacological doses. Therefore, there is a gap in the knowledge about the ability of whole foods consumed at more physiological levels to suppress inflammation and reduce adiposity. Additionally, research has demonstrated that the bioavailability of many polyphenols is low [11].

Thus, microbes in the lower intestinal tract are exposed to these non-absorbed phytochemicals. Such observations have caused many to speculate that the gut microbiome may play an important role in eliciting these beneficial effects of dietary polyphenols [12].

Influence of Gut Microbes on Obesity and Inflammation

Obesity is associated with alterations in gut microbiota. High-fat and high-caloric diets have been shown to increase Gram-negative bacterial colonization of the intestine, resulting in increased plasma lipopolysaccharide (LPS) levels associated with metabolic endotoxemia [12]. For example, obesity resulted in increases in the relative abundance of *Firmicutes spp.* (Gram negative) and the reduction of *Bacteroidetes spp.* (Gram positive) in the gut of genetically obese mice [13]. When examining fecal samples from lean and obese individuals, the ratio of *Firmicutes* to *Bacteriodetes* was greatest in the obese group [14]. *Akkermansia muciniphila*, a beneficial bacteria associated with mucin-degradation and improved gut barrier function, has been found to be greatly decreased in genetically and diet-induced obese mice. Furthermore, prebiotic treatment with oligofructose restored the abundance of *A. muciniphila*, while also improving gut barrier integrity, reducing body weight gain, fat mass development, and fasting hyperglycemia [15]. It has also been demonstrated that supplementation of a polyphenol-rich cranberry extract results in increases in the abundance of *A. muciniphila* , which in turn was associated with protection from diet-induced obesity, insulin resistance, and intestinal inflammation in mice fed a high fat diet [16].

Recently, the linkage between consumption of a diet high in saturated fat and the prevalence of deleterious sulfate-reducing bacteria (SRB) has been explored. For example, Apoa-1 knockout mice with increased adiposity and impaired glucose disposal and wild-type mice were fed a high-fat, lard-based diet (60% kcal from fat) for 25 weeks [17]. It was observed that compared to low-fat fed control mice, both genetic mouse models fed the high fat diet developed significant alterations in the microbial community within the gut. These alterations included reductions in beneficial *Bifidobacterium spp.* and increased abundance of *Desulfovibrionaceae* [18], a group of SRB that produce hydrogen sulfide, a genotoxic gas causing gut barrier dysfunction and endotoxemia [reviewed in 19]. Additionally, feeding IL-10 knockout mice a high milk fat-based diet (37% kcal from butter fat) decreased populations of gut barrier-protective bacterial species and increased SRB [19]. This increase in SRB directly correlated with the degree of expression of markers of inflammatory bowel disease and gut inflammation. These two studies revealed the drastic alterations in gut bacterial species, specifically increases in genotoxic SRB, with consumption of a diet rich in saturated fat [19].

Anti-inflammatory and Anti-obesogenic Properties of Grapes

Grape and specific components found in grapes have been shown to impact inflammation as well as gut microbiota. Grapes are rich in polyphenols, particularly resveratrol and flavonoids. The polyphenol content of grapes is shown in Table 1 [reviewed in 20]. Resveratrol consumption has been shown to reduce adiposity and inflammation [21], as well as insulin resistance and diabetes [22]. Some of the

mechanisms behind these actions are thought to involve activation of sirtuin (SIRT1)1, and ERK and suppression of the TLR2/4 and NFkB pathways [23]. It has been observed that resveratrol increases fatty acid oxidation through increases in expression of carnitine palmitoyl transferase (CPT), which may be responsible for the reductions in adiposity discussed previously [24].

Anthocyanidins are a group of flavonoids and are present in large amounts relative to other polyphenols in grapes. These compounds are found attached to sugar groups, typically 3-O-glucosides, and are referred to as anthocyanins. The most abundant anthocyanins in grapes are malvidin / malvidin 3-O-glucoside and cyanidin / cyanidin 3-O-glucoside [reviewed in 20]. Cyanidin 3-O-glucoside has been shown to improve insulin sensitivity and hyperglycemia in diabetic mice through the down-regulation of retinol binding protein (RBP) 4 [24], improve obesity and triglyceride metabolism by regulating lipoprotein lipase activity in KK-Ay mice [25], and improve insulin resistance thorough regulation of GLUT4 gene expression [26]. In vitro, malvidin treatment of RAW264.7 macrophages attenuated LPS- induced NFκB and MAPK activation, reactive oxygen species production, and mitochondrial depolarization while stimulating Akt (protein kinase B) activation, which is associated with improved insulin-stimulated glucose uptake [27]. In vitro, 3T3-L1 preadipocytes exposed to anthocyanins resulted in dose-dependent reductions in triglyceride accumulation as well as reductions in gene and protein expression of the transcription factors liver x receptor alpha (LXRα), stearoyl regulatory element binding protein 1c (SREBP1c), peroxisome proliferator-activated receptor-gamma (PPARγ), and CAAT enhancer binding protein alpha (C/EBPα) and their

target genes associated with lipogenesis including fatty acid synthase (FAS), stearoyl CoA desaturase 1 (SCD1), acetyl CoA carboxylase (ACC) [28].

In vivo, Zucker rats fed a high fat diet supplemented with grape seed procyanidins had lower WAT mRNA levels of tumor necrosis factor (TNF)α, IL-6, and C-reactive protein (CRP) and lower plasma levels of CRP compared to high-fat-fed control rats [29]. Consumption of a polyphenol rich grape extract prevented high fat diet-induced obesity by increasing in adiponectin secretion and reducing oxidative stress [30]. Additionally, our lab has shown that California table grape supplementation decreased insulin resistance acutely, and systemic inflammation chronically in mice fed an extremely high fat, lard-based diet (e.g., 60% kcals from lard;) [31].

Although the above studies indicate that plant polyphenols have beneficial health effects, these compounds have low bioavailability (i.e., less than 5% are absorbed in the upper intestinal tract, and thus the majority of ingested polyphenols reach the cecum and colon [reviewed in 11]. Therefore, we hypothesized that gut microbiota may be involved in mediating the beneficial systemic effects associated with grape consumption.

Prebiotic Properties of Grapes

A prebiotic is defined as "the selective stimulation of growth and/or acitivity(ies) of one or a limited number of microbiota genus(era) species in the gut microbiota that confer(s) health benefits to the host" [32]. Grapes contain many components that have the potential to function as prebiotics. For example, it has been demonstrated that feeding grape antioxidant dietary fiber, containing both the fiber and antioxidant

components from grapes, significantly increased *Lactobacillus spp*. within in the cecum of rats compared to controls [33]. Feeding broiler chicks a diet containing grape pomace or grape seed extract resulted in increases in ileal populations of *Enterococcus spp.*, a beneficial bacterial species, while decreasing the concentrations of *Clostridium spp.*, a pathogenic species [34].

It has been shown that certain fibers are metabolized by gut microbes and result in the production of short chain fatty acid (SCFA)s. This production of SCFAs, which includes butyrate, propionate, and acetate, has been associated with improvements in intestinal and systemic health. For example, butyrate plays an integral role in the function and integrity of colonic cells. Colonic cells use butyrate as a preferred energy source and preferentially do so even in the presence of other substrates such as glutamine and glucose. Intestinal SCFA production also has the potential to inhibit the growth of pathogenic organisms through the mechanism of reducing the pH of both the lumen and feces. Additionally, butyrate has been shown to regulate gene expression, differentiation, and apoptosis of colonocytes and may provide a protective mechanism against colon carcinoma cells [reviewed in 35]. Through this specific inhibition of pathogenic organisms, the beneficial organisms residing in the colon are better able to thrive and may then be able to exert greater benefits. Additionally, SCFAs have been associated with improvements in host metabolism [36]. For example, SCFAs produced via microbial fermentation has been demonstrated to activate the SCFA receptors GPR41 and 43 in WAT, which is associated with reduced fat accumulation. Activation of these receptors also increases secretion of glucagon-like peptide 1 and 2 (GLP-1 and -2 resulting in increased insulin secretion and

sensitivity within muscle and liver [37]. Activation of GPR43 is also associated with reductions in inflammatory responses associated with consumption of a high fat diet [38].

In contrast to butyrate, acetate and propionate can increase energy harvest via increased absorption of these two SCFA. For example, For example, propionate is a precursor for hepatic gluconeogenesis, propionate and acetate are precursors of cholesterol synthesis, and acetate and butyrate are substrates for hepatic and WAT triglyceride synthesis. Average molar ratios of acetate, propionate, and butyrate are 60: 23: 17 under normal feeding conditions. However, these ratios can change greatly depending on diet composition, particular the due to the amounts and types of dietary fiber [38].

While numerous research studies have shown that the prebiotic potential of certain foods to be highly dependent on the specific fiber content [reviewed in 35; reviewed in 36], less is known about the capacity of the polyphenols in these same foods to elicit a prebiotic response. Because of this, recent research has focused on examining specific phenolic compounds in relationship to gut microbiota and colon health.

Studies that examine microbial metabolism of anthocyanins in the colon are scarce. However, research has shown that only a small fraction of dietary anthocyanins is absorbed in the upper intestinal tract, and therefore large amounts of these polyphenols are exposed to the microbial community in the cecum and colon, thereby impacting microbial growth and metabolism. [40]. The most abundant metabolites produced from anthocyanins include gallic acid and syringic acid, both of which have been shown to have antioxidant activities. Syringic acid has also been shown to inhibit cyclooxygenase

(COX-2), a enzyme catalyzing the conversion of arachidonic acid to prostanoids. Gallic and syringic acid have also been detected in large concentrations within the gut as well as the blood [41]. Collectively, these data indicate that anthocyanins and their metabolites may be responsible for some of the proposed health benefits associated with grape consumption.

Summary of Published Data

Given the obesity epidemic, it is important to identify strategies to prevent and treat obesity. The current literature indicates that genetic and diet-induced obesity is associated with chronic, low-grade inflammation systemically as well as within the gut [4-7]. Polyphenols, which are abundant in grapes, have been shown to have many anti-inflammatory and anti-oxidant properties [8-11]. However, the mechanisms behind these actions are still unclear. With diet-induced obesity, the populations of deleterious gram-negative bacteria are more abundant than other beneficial bacterial species [13-16]. It has been suggested that these alterations in bacteria may play a role in initiating the inflammatory response within the gut, leading to the release of proinflammatory compounds (e.g., LPS) that enter the bloodstream and cause systemic health issues. Several research studies have shown that grape products or components increase beneficial microbial species within the gut such as *Lactobacillus spp* and *Enterococcus spp* [33, 34]. A polyphenol-rich extract from cranberries, similar in polyphenol composition to grapes, has been shown to increase the abundance of beneficial bacterium *A. muciniphila* resulting in improvements in the metabolic profile of obese mice [16].

Additionally, reductions in systemic markers of inflammation with feeding or supplementing grape products or their components have been observed [29]. Also, grapes contain many indigestible components such as anthocyanins and fiber that have been shown to reach the colon and subsequently be metabolized by the intestinal bacteria to yield beneficial metabolites such as gallic and syringic acid that possess antioxidant functions [40, 41]. These indigestible components may also impact SCFA production via bacterial fermentation which is associated changes in energy harvest and with activation of GPR41 and 43, depending on the type of SCFA produced, resulting in improvements in insulin sensitivity and host energy homeostasis [37, 38,39].

Based on these data, I proposed the *central hypothesis* that California table grapes, and one or more of their bioactive fractions, would decrease adiposity, systemic inflammation, and insulin resistance associated with diet-induced obesity. I predicted these beneficial systemic outcomes would be due, in part, to modulation of the gut microbiota and subsequent improvements in intestinal health.

To test my central hypothesis, the following *specific aims* were pursued:

Aim #1. Determine the extent to which California table grapes attenuate body fat accumulation, systemic inflammation, and insulin resistance, as well as impact gut microbiota in mice fed an American type diet rich in saturated fat from butter; and

Aim #2. Identify the key bioactive fractions in California table grapes responsible for reducing adiposity, inflammation, and insulin resistance, and modulating gut microbiota

in mice fed an American type diet rich in saturated fat from four sources. These specific aims were be pursued in the following two chapters.

Table 2.1. The Polyphenol Composition of Powdered Grapes (PG).

Polyphenols	Compounds	Content (mg/kg PG)
Anthocyanins	Malvidin	145.2
	Cyanidin	125.0
	Peonidin	31.7
Flavonols	Quercetin	32.6
	Isorhamnetin	6.8
	Kaempferol	5.6
Flavan-3-ols	Catechin	19.7
	Epicatechin	12.6
Stilbenes	Resveratrol	1.75

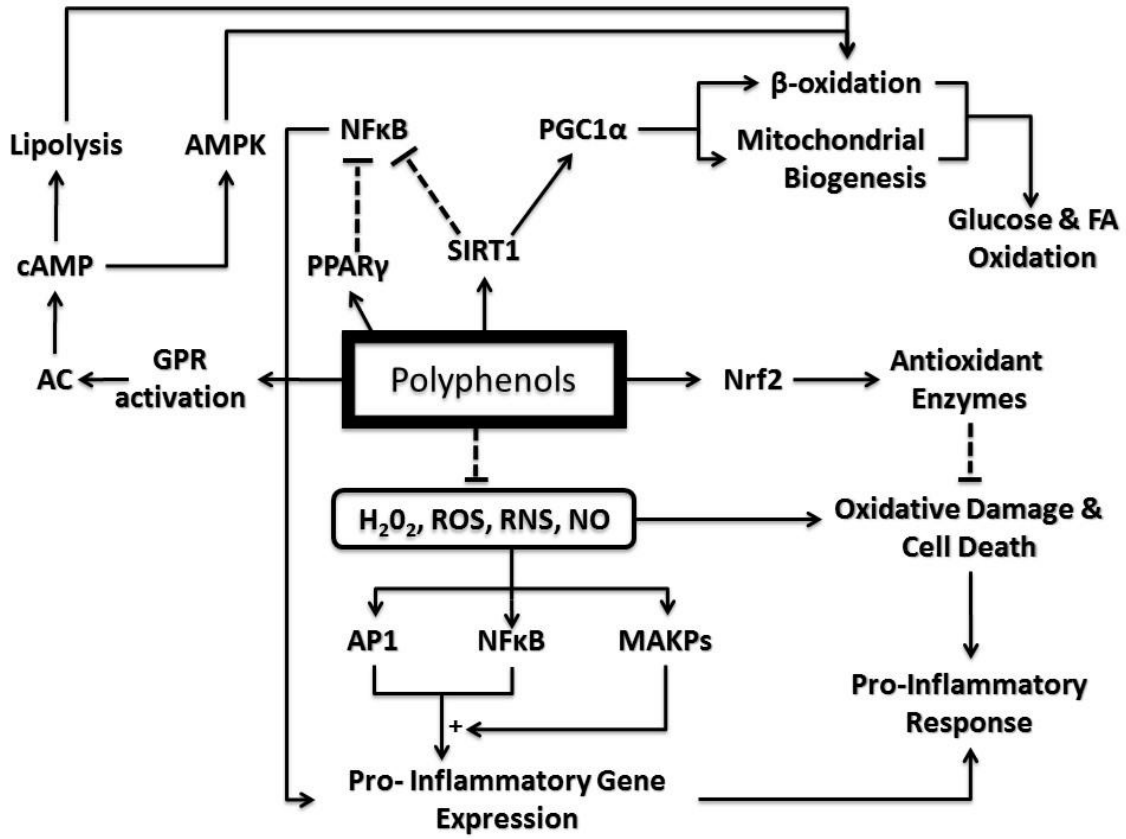

Figure 2.1. **Anti-Inflammatory and Anti-Oxidant Effects of Polyphenols in Obesity.** Polyphenols have been shown to inhibit the production of pro-oxidant compounds including hydrogen peroxide (H202) reactive oxygen species (ROS), reactive nitrogen species (RNS), and nitric oxide (NO) thereby preventing activation of the MAPK, NFkB, and AP1 pathways and subsequent proinflammatory response. Additionally, polyphenols have been shown to activate nuclear factor erythroid 2-related factor 2 (Nrf2), which increases the expression of antioxidant enzymes that inhibit oxidative damage and cell death associated with pro-oxidant compounds. Polyphenols also may activate peroxisome proliferator-activated receptor gamma (PPARγ) and sirtuin 1 (Sirt1) which also inhibit NFκb and pro-inflammatory responses. Sirt1 also can activate peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC1α) which stimulates beta-oxidation and mitochondrial biogenesis, leading to glucose and fatty acid (FA) oxidation. Additionally, polyphenols may activate G-protein receptors (GPR) stimulating adenylate

cyclase (AC), cyclic adenosine monophosphate (cAMP), and 5’ adenosine monophosphate activated protein kinase (AMPK) which stimulate lipolysis, beta-oxidation, mitochondrial biogenesis and subsequent glucose and fatty acid oxidation.
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CHAPTER III

CALIFORNIA TABLE GRAPE CONSUMPTION REDUCES ADIPOSITY, HEPATIC TRIGLYCERIDES, LIPOGENIC GENE EXPRESSION, AND ABUNDANCE OF SULFIDOGENIC BACTERIA IN MICE FED BUTTER FAT

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Abstract

The objective of this study was to examine the extent to which consuming polyphenol-rich, California table grapes reduces adiposity, hepatic steatosis, markers of inflammation or lipid metabolism, or impacts gut microbiota in mice fed a butter-rich diet. Male, C57BL/6J mice were fed a low fat diet or butter-rich diet with or without 3% or 5% grapes for 11 weeks. Total body and inguinal fat content were reduced in mice fed both levels of grapes compared to their high-fat, sugar controls. Mice fed 5% grapes had lower liver weights and triglyceride levels, and decreased hepatic expression of lipogenic glycerol-3-phosphate acyltransferase *(Gpat1)* compared to 5% controls. Mice fed 3% grapes had lower hepatic mRNA levels of the lipogenic genes peroxisome proliferator-activated receptor gamma 2, sterol-CoA desaturase 1, fatty-acid binding protein 4, and *Gpat1* compared to 3% controls. In white adipose tissue (WAT), mice fed 5% grapes had

decreased mRNA levels of the lipogenic gene acylglycerol-3-phosphate-O-acyltransferase 2 compared to controls. Although grape feeding had only a minor impact on markers of inflammation in WAT or intestine, 3% grapes decreased the intestinal abundance of sulfidogenic *Desulfobacter* spp., and the *Bilophila wadsworthia*-specific dissimilatory sulfite reductase gene, and tended to increase the abundance of the beneficial bacterium *Akkermansia muciniphilia* compared to controls. In addition, via 16s rRNA sequencing of cecum mucosa, Bifidobacterium, Lactobacillus, Allobaculum, and other genera were found to be negatively correlated with body fat percentage and inguinal fat weight. Allobaculum in particular was increased in both the LF and 3% grapes groups. Notably, grape feeding attenuated the high-fat induced impairment in localization of the intestinal tight junction protein zonula occludens. Collectively, these data indicate that some of the adverse health consequences of consuming a diet rich in saturated fat can be attenuated by table grape consumption.

Introduction

Currently 35.9% of adults and 17% of youth in the United States have been diagnosed as obese [1]. Obesity is also a global health condition affecting more than 10% of adults worldwide [2]. Of growing concern is the association between the rise in obesity and chronic inflammatory conditions such as type 2 diabetes, hypertension, and cardiovascular disease [3]. Physiologically, obesity is the result of an expansion of white adipose tissue (WAT) which typically elicits inflammatory signals involved in the recruitment of macrophages and other immune cells into the WAT. This results in a

proliferation of circulating and tissue levels of proinflammatory cytokines and chemokines [4]. While increased tissue levels of these proinflammatory agents perpetuates the inflammatory cycle, those released systemically impair glucose disposal and lipid metabolism and hemostasis that contribute to the development of metabolic diseases. However, the exact mechanisms which initiate WAT inflammation resulting from diet-induced obesity remain unclear.

Gut microbes have received much attention due to their potential involvement in the development of obesity [5], chronic inflammation [6-8], and insulin resistance [9]. Diets high in fat, particularly those rich in saturated fatty acids found in milk fat [10], have been implicated in the reduction of gut barrier-protecting bacteria as well as increasing the abundance of deleterious bacteria including sulfidogenic bacteria [reviewed in 11]. A correlation exists between the extent of these changes in microbiota and the degree of obesity and insulin resistance in test subjects [12]. Furthermore, the main byproduct of sulfidogenic bacteria like *Bilophila wadsworthia* and *Desulfovibrionaceae* spp. is hydrogen sulfide gas, which is genotoxic and cytotoxic and positively correlated with development of ulcerative colitis, gut inflammation, irritable bowel syndrome, and colon cancer [10, 13, 14].

In contrast, changes to the intestinal microbiome can have beneficial effects. Indeed, research models have demonstrated that elimination of gut microbes [5-9, 11, 12, 15] or inoculation with specific pre- (e.g., non-digestible sugars, fiber, or polyphenols) or probiotics (e.g., *Lactobacillus acidophilus, Bifidobacterium* spp., *Akkermansia muciniphila, Clostridium trybutyricum*) [reviewed in 11, 16-19] can attenuate obesity or

metabolic dysfunction. Polyphenols found in fruits and vegetables [20-22] are of particular interest as they are poorly absorbed in the upper gastrointestinal tract, and thus persist in the distal small intestine, cecum, and colon [23] where they may influence microbiota taxa and their metabolites [24]. In addition, their anti-inflammatory, anti-oxidant, or anti-microbial actions have been reported to positively influence gut microbes and inflammation [reviewed in 25].

Grapes are rich in polyphenols including anthocyanins [reviewed in 25], and thus may have beneficial effects on intestinal or systemic inflammation. The anti-inflammatory and anti-oxidant properties of California table grapes have been demonstrated in rats supplemented with 9 human servings (3%, w/w) of powdered table grapes for 18 weeks, resulting in lowered blood pressure, improved cardiac function, and reduced systemic inflammation and oxidative damage [26]. These effects correlated with increased cardiac peroxisome proliferator-activated receptor (PPAR)α/γ activity and decreased nuclear factor kappa B (NF-κB) activity, along with reductions in cytokine levels [27]. Similarly, table grape-mediated reductions in atherosclerotic lesion areas were associated with increased serum antioxidant status and decreased macrophage-mediated oxidation of low density lipoproteins (LDL) [28]. Additionally, when women supplemented with powdered California table grapes (36 g/d) their plasma lipid profile improved and plasma (e.g., tumor necrosis factor alpha, TNFα) or urine (e.g., prostaglandin F2 alpha) markers of inflammation and oxidative stress were reduced [29]. Consistent with these data, we demonstrated that C57BL/6J mice fed a high fat diet (60% kcals from lard) supplemented with powdered California table grapes (3%, w/w), improved glucose tolerance at 5

weeks, and decreased markers of inflammation ≈20-50% in serum and WAT at 18 weeks without affecting body fat levels or food intake [30].

Collectively, these data indicate that; (i) gut microbiota are influenced by diets abundant in saturated fats such as butter fat, in a manner that impacts gut inflammation and barrier function, and systemic inflammation, (ii) non-digestible carbohydrates and polyphenols positively impact adverse outcomes caused by high fat-feeding, and (iii) grape consumption can attenuate inflammation and oxidative stress in several animal models and in humans. However, little is known about how California table grapes decrease chronic inflammation associated with saturated fat-feeding and obesity. Therefore, the objective of this study was to examine the extent to which consuming California table grapes reduces adiposity, hepatic steatosis, markers of inflammation or lipid metabolism, or impacts gut microbiota in mice fed a butter-rich diet.

Materials and Methods

Animals

Four-week old, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and acclimated on a standard chow diet for 1 week. Mice were housed in pairs, maintained at a temperature of 22˚C with 50% humidity, and exposed to a 12 h light/ 12 h dark cycle. Mice received food and water *ad libitum* and measures of body weight and food intake were conducted once and twice per week, respectively. All experimental procedures were performed under ethical standards and approved by the

Institutional Animal Care and Use Committee of the University of North Carolina at Greensboro.

Diets

Animals were randomly assigned to one of five dietary treatments (n=10 per treatment group) as follows: low fat (LF; 10% of energy from fat), high fat (HF; 34% of energy from fat) plus 3% powdered grapes (w/w; HF-3G), high fat plus 3% sugar (w/w; HF-3S), high fat plus 5% powdered grapes (HF-5G) and high fat plus 5% sugar (HF-5S). The high fat diets consisted of approximately 3% energy from soybean oil and 31% energy from butter. Thus, the HF diets were rich in fat, especially saturated fat, and mimicked the average calories from fat in an American-type diet. The lyophilized (i.e., powdered) California table grapes, kindly provided by the California Table Grape Commission consisted of a mixture of red, green, and purple seeded and seedless grapes. The 3% and 5% dietary levels of grapes were comparable to 9 and 15 human servings (1 serving is equivalent to 1 cup of whole berries) of grapes, respectively. The HF-3S and HF-5S diets consisted of a mixture of fructose and glucose to control for the natural sugar content of the powdered grape diets. Detailed composition of the diets is illustrated in **Table 3.1**.

Intraperitoneal glucose tolerance tests (GTT) and fasting insulin levels

Intraperitoneal (i.p.) GTT were performed on weeks 3, 6, and 9 on non-anesthetized mice. Mice were fasted for 8 h and given an i.p. injection of glucose (i.e., 20% solution at 1 g/kg body weight). Blood from the tail vein collected at baseline and 5, 15, 30, 60, and

120 minutes post-i.p. glucose injection was used to quantify glucose levels using a Bayer Contour blood glucose monitor and strips (Bayer Healthcare, Tarrytown, NY, USA). Plasma insulin levels were detected using an ultrasensitive mouse insulin kit (Crystal Chem, Inc, Downers Grove, IL). The homeostasis model assessment method (HOMA) for insulin resistance (IR) was used employing the following formula: [fasting insulin concentration (ng/ml) x 24 x fasting glucose concentration (mg/dl)] / 405 [16].

Body fat measurements via Dual X-Ray Absorptiometry (DEXA)

Percent body fat was measured using DEXA on a GE Lunar Prodigy Advanced System (GE Healthcare, Milwaukee, WI) at weeks 5 and 10. During the measurement, mice were lightly anesthetized with isoflurane using a SomnoSuite Small Animal Anesthesia System with Integrated Digital Vaporizer isoflurane system. Measurements were taken in duplicate to reduce the possibility of error and values expressed are an average of the two measurements.

Tissue collection

After 11 weeks of dietary intervention, mice were fasted for 8 h and euthanized via isoflurane-induced anesthesia followed by decapitation. Plasma was collected at time of harvest. Four white adipose tissue (WAT) depots were collected; epididymal, mesenteric, inguinal, and retroperitoneal. Additionally, livers were harvested and intestinal mucosa and digesta were collected from the duodenum, jejunum, ileum, cecum, and proximal and distal colon. Weights of the WAT depots and liver were recorded and all collected

samples were immediately snap-frozen in liquid nitrogen and stored at -80^{o}C until analysis.

Liver and serum triglyceride (TG) levels

Liver TG content was measured as previously described [31]. Plasma TG content was determined using a commercial assay from Thermo Scientific and was conducted following the manufacturer's protocol (Infinity TG assay #TR22421 and TG standards #TG22923; Norcross, GA).

RNA extraction and qPCR

Adipose and intestinal samples were homogenized in QIAsol reagent and total RNA was extracted using QIAgen mini lipid kit obtained from Qiagen (Valencia, CA). For hepatic samples, a QIAgen mini universal kit from Qiagen was used. The quality and concentration of RNA were examined using absorbance at 260 nm and integrity determined using the absorbance ratio of 260/280 on a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA was created by reverse transcription using 1 ug of RNA and a high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocols. qPCR was performed in a 7500 FAST Real Time PCR system (Applied Biosystems). The expression of different genes related to inflammation, lipogenesis, and lipolysis in WAT depots and liver were measured using Taqman Gene expression assays purchased from

Applied Biosystems. TATA-binding protein (*Tbp*) was the endogenous reference gene utilized for all assays and fold differences in gene expression were calculated as $2^{-\Delta\Delta Ct}$.

Immunoblotting

Immunoblotting was conducted as previously described [31] using primary antibodies for sterol-CoA desaturase-1 (#2283S; SCD1, Cell Signaling), carnitine palmitoyltransferase 1A (#12252S; CPT1a, Cell Signaling), peroxisome proliferator-activated receptor gamma (#2443S; PPARγ Cell Signaling), β-actin (#4967; Cell Signaling), proliferator-activated receptor alpha (#sc9000; PPARα, Santa Cruz Biotechnology Inc., Santa Cruz, CA), glycerol-3-phosphate acyltransferase (#sc382257; GPAM, Santa Cruz), and sterol regulatory element-binding protein 1C (#sc366; SREBP1c, Santa Cruz) all at dilutions of 1:1000. Adipocyte fatty acid binding protein (aP2) was kindly provided by Dr. David Bernlohr (U. of Minnesota) and used at a 1:10,000 dilution. Horseradish peroxidase-conjugated secondary antibodies were probed for 2 h at room temperate at 1:1000 dilutions. Blots were exposed to a chemiluminescence reagent and X-ray films were developed using a SRX-101A Konica Minolta film developer.

Barrier function in ileum

The localization of the tight junction protein zonula occludens-1 (ZO-1) was determined in ileum samples that had been embedded in Tissue-Tek Cyro-OCT

compound, sliced in 5 mm sections, fixed in cold methanol, and incubated with a polyclonal rabbit anti-ZO-1 antibody as previously described [32].

PCR amplification of 16S rRNA and functional gene targets

Real-time quantitative PCR (qPCR) was performed with a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). 16S rRNA gene-specific primers were used to target specific bacterial genera; *Akkermansia muciniphila*, *Bilophila wadsworthia* (F-AAGTCCTTCGGGGCGAGTAA) (R-ATCCTCTCAGACCGGCTAC), *Desulfovibrio* spp. (DSV), *Desulfobulbus* spp. (DBB), *Desulfobacter* spp. (DSB), and *Desulfotomaculum* spp. (DFM) [4]. The *B. wadsworthia*-specific dissimilatory sulfite reductase (*dsrA-Bw*) [20], which encodes an enzyme that catalyzes a step in the reduction of inorganic sulfate to hydrogen sulfide, was targeted to measure the abundance of this member of the Desulfovibrionaceae family. Four functional genes utilized by *Fusobacterium nucleatum* in the fermentation of cysteine were measured; i.e., two homologues of L-cysteine desulfhydrase (FN0625 and FN1220), Cystathionine-β synthase (FN1055), and L-methionine-γ-lyase (FN1419). The abundance of butyrate-producing bacteria was also measured using the degenerate primer Butyrl-CoA transferase. Standard curves were constructed using cloned 16S rRNA and functional genes or amplified PCR product. Genomic DNA extraction and PCR experiments were conducted as previously described [4].

Sequencing of 16s rRNA gene using Illumina Mi-Seq platform

To assess bacterial community structure, primers specific for 16S rRNA V4-V5 region (Forward: 338F: 5'-GTGCCAGCMGCCGCGGTAA-3' and Reverse: 806R: 5'-GGACTACHVGGGTWTCTAAT-3') that contained Illumina 3' adapter sequences as well as a 12-bp barcode were used. Sequences were generated by an Illumina MiSeq DNA platform at Argonne National Laboratory and analyzed by the program Quantitative Insights Into Microbial Ecology (QIIME) [33]. Operational Taxonomic Units (OTUs) were picked at 97% sequence identity using open reference OTU picking against the Greengenes database (05/13 release) [34]. OTUs were quality filtered based on default parameters set in the open-reference OTU command in QIIME and sequences were rarified to an equal sampling depth of 15000 reads per sample. Representative sequences were aligned via PyNAST [33], taxonomy assigned using the RDP Classifier [35], and a phylogenetic tree was built using FastTree [36]. Beta Diversity is represented by measuring UniFrac distances calculated using both weighted and unweighted algorithms and visualized with PCoA plots generated in Emperor. Prior to statistical analyses, OTUs occurring in less than 50% of samples were filtered from the OTU table. Significant changes in OTU abundance were assessed using Kruskal Wallis test (FDR correction $p \leq 0.05$). Multivariate statistical tests include ADONIS, ANOSIM, and PERMANOVA tests [33]. Spearman correlations and Principal Component Analysis (PCR) were run using MATLAB software.

Statistical Analysis

Data were analyzed using a one-way ANOVA and Student's *t* test to compute individual pairwise comparisons of means ($p<0.05$). We also used Bonferroni's posthoc test to perform specific comparisons where appropriate. Analyses were conducted using the JMP software program version 10.0 for Windows (SAS, Cary, NC). Relative abundances generated from 16s rRNA sequencing were analyzed in GraphPad Prism Version 6 using ANOVA followed by Dunn's test for multiple comparisons. Data are expressed as means + S.E.M.

Results

Grape consumption decreases body fat

Body weight gains and energy intakes were greater in all HF-fed mice compared to the LF control mice (**Table 3.2**). Mice fed powdered grapes (HF-3G, HF-5G) had similar body weights and energy intakes compared to their HF-sugar controls (HF-3S, HF-5S; Table 3.2). Mice fed the HF-sugar control diets had greater body fat percentage and total WAT depot weights compared to the LF controls (**Fig. 3.1**). Mice fed the HF-3G diet had lower percent body fat at week 5 and mice fed powdered grapes at both levels had lower body fat percentages and inguinal fat depot weights at week 10 compared to their respective HF-sugar controls (Fig. 3.1). The HF-3G diet had lower total WAT depot weights compared to HF-3S controls (Fig. 3.1).

GTT, fasting glucose, insulin, and TG levels, and HOMA-IR scores unaffected by grapes

To assess the impact of grape consumption on insulin resistance and glucose sensitivity, GTT were conducted at weeks 3, 6, and 10, and fasting plasma glucose and insulin and HOMA-IR were measured at week 11. Mice consuming the HF-sugar control diets had impaired GTT at all three time points compared to the LF controls (**Fig. 3.2**). However, consuming grapes did not significantly improve GTT, fasting insulin, glucose, serum TG levels, or HOMA-IR scores.

Grape consumption lowers hepatic TG levels and the expression of several lipogenic genes

Liver tissue was analyzed to assess the impact of grape consumption on liver TG levels and markers of lipogenesis and fatty acid oxidation. Mice consuming the HF-sugar control diets had greater liver weights, TG levels (HF-5S only), and mRNA levels of the lipogenic genes *Pparγ2*, *Scd1* (HF-5S only), *Srebp1c*, cluster of differentiation 36 (*Cd36*; HF-5S only), and glycerol-3-phosphate acyltransferase 2 (*Gpat2*; HF-3S only) compared to the LF-fed mice (**Fig. 3.3**). Mice fed the HF-3G diet had decreased mRNA levels of *Pparγ2*, *Scd1*, fatty acid binding protein 4 (*Fabp4*), and *Gpat1* compared to the HF-3S controls. Mice fed the HF-5G diet had decreased TG levels and mRNA levels of *Gpat1* compared to the HF-5S controls. None of the genes associated with fatty acid oxidation (e.g., *Pparα*, *Cpt1a*, acyl-CoA oxidase 1 (*Acox1*)) were impacted by grape feeding. Consuming grapes had no impact on the protein levels of *Pparγ*, *Scd1*, *Pparα*, *Fabp*, *Cpt1a*, or *Gpam* (data not shown).

Grape consumption differentially impacts WAT genes associated with inflammation and lipid metabolism

To determine whether insulin resistance caused by HF-feeding was due to WAT inflammation, inguinal (subcutaneous), epididymal (visceral), and mesenteric (visceral) WAT mRNA levels for several proinflammatory genes (i.e., cluster of differentiation 11c (*Cd11c*), epidermal growth factor-like module containing mucin-like hormone receptor 1 (*Erm1*; F4/80 human orthologue), monocyte chemoattractant protein 1 (*Mcp1),* *Tnfα*, Toll-like receptor 4 (*Tlr4),* and interleukin 6 (*Il6*) were measured. In epididymal WAT, mice fed the HF-sugar controls had increased mRNA levels of *Tnfα* (HF-5S), *Cd11c* (HF-5S), *Il6,* and *Mcp1* (HF-5S) compared to the LF controls (**Fig. 3.4A**). Grape feeding had no impact on these genes. In inguinal depot WAT, only mice fed the HF-5S diet had increased inflammatory gene expression (i.e., *Mcp1*), which was decreased in mice fed the HF-5G diet (Fig. 3.4A). In mesenteric WAT, inflammatory gene expression was not increased by HF-feeding.

To determine if the reduction in adiposity by grape consumption was due to alterations in the expression of genes associated with fat synthesis or oxidation, epididymal and inguinal WAT were analyzed for mRNA markers of; (i) lipogenesis (i.e., *Pparγ2*, *Srebp1c*, *Scd1*, acylglycerol-3-phosphate-O-acyltransferase 2 (*Agpat2*), fatty acid synthase (*Fas*), acetyl-CoA carboxylase (*Acc*), perilipin 1 (*Plin1*), *Gpat1*), (ii) lipolysis (i.e., adipose TG lipase (*Atgl*); and (iii) beta-oxidation (i.e., Acox1, *Cpt1b*, *Pparα*). In epididymal WAT, the mRNA levels of the lipogenic genes *Pparγ2*, *Agpat2*, and *Scd1* (HF-5S) were higher in the HF-sugar controls compared to the LF-controls, and similar to

the HF-grape groups (**Fig. 3.4B**). In inguinal WAT, the mRNA levels of the lipogenic genes *Srebp1c* (HF-3S), *Scd1*, *Agpat*2 (HF-5S), *Fas* (HF-5S), and *Gpat2* (HF-5S) were higher in the HF-sugar controls compared to the LF-controls (**Fig. 3.4C**). Mice fed the HF-5G diets had lower mRNA levels of *Agpat2* compared to HF-5S control. The mRNA levels of the fatty-acid oxidizing genes *Pparα* and *Cpt1b*, and the lipolytic gene *Atgl* (HF-3S) were lower in the HF-sugar controls compared to the LF control, and similar to the HF-grape groups.

Minimal influence of HF-feeding and grape consumption on markers of intestinal inflammation and barrier function

Given the reported adverse effects of consuming saturated fats [reviewed in 11], particularly from milk fat [10], on intestinal health, and potential prebiotic impact of grapes, we measured the effects of our diets on markers of intestinal inflammation and barrier function. For inflammatory status, the mRNA levels of *Cd11c*, *Erm1*, *Mcp1*, *Tnfα*, *Tlr4*, and *Il6* in ileum and distal colon mucosa and the activity of duodenal alkaline phosphatase and ileal myeloperoxidase were measured. Surprisingly, the only proinflammatory gene increased in the ileal mucosa of HF-sugar fed mice was *Tnfα*, which was similar to the HF-5G group (**Fig. 3.5A**). None of the proinflammatory genes measured in the colonic mucosa where increased by HF-sugar feeding or grape consumption. Similarly, the activities of alkaline phosphatase and myeloperoxidase were not influenced by HF-sugar feeding or grape consumption (data not shown).

To assess intestinal barrier function, the mRNA levels of the tight junction proteins zonula occludens (*Zo1*), claudin-1, and occludin-1 and the localization of ZO-1 at the apical surface of the ileal epithelium were measured. Although the ileum mucosal gene expression of these tight junction proteins was not impacted by the diets, the localization of ZO-1 was impaired in the HF-sugar control diets compared to the LF controls, and improved by grape feeding (**Fig. 3.5B**).

16s rRNA sequencing of the gut microbiota reveals genera associated with body fat percentage and inguinal fat pad weight

Given the reported adverse effects of consuming saturated fats [reviewed in 11], particularly from milk fat [10], on intestinal microbes, we measured the effects of our diets on the abundance of several mucosal sulfidogenic bacteria (i.e., *DBB, DSB, dsrA, DFM, DSV*, and *B. wadsworthia*) or their gene products (i.e., *B. wadsworthia* specific *dsrA-Bw*, *Fusobacterium nucleatum* functional genes including two L-cysteine desulfyhdrases (*FN0625* and *FN1220*) cystathionine-β synthase (*FN1055*), and L-methionine-γ-lyase (*FN1419*)). Although HF-feeding did not increase the expression of any of these genes associated with sulfur metabolism in ileum or colon mucosa compared to LF control mice (data not shown), mice consuming the HF-3G diet had decreased mRNA levels of *DSB* and *dsrA-Bw* expression in ileum mucosa compared to mice consuming the HF-3S diet (**Fig. 3.6A**).

We also measured the expression of a functional gene (i.e., butyrl-CoA transferase) of health- promoting, butyrate-producing bacteria and on the abundance of *Akkermansia*

muciniphila, a mucin-degrading bacteria associated with prebiotic-mediated reduction in obesity [37]. Although not statistically significant, mice consuming grapes had increased levels of *Akkermansia muciniphila* in the cecum digesta (HF-3G), colon digesta (HF-3G), and proximal colon mucosa (HF-5G) compared to their HF-sugar controls (**Fig. 3.6B**).

Sequencing of the 16s rRNA gene reveals alterations in microbial structure and relationships

In order to better understand the impact of grape feeding on gut bacterial community structure, an untargeted approach was used by sequencing the 16s rRNA gene in cecum mucosal samples. Sequencing was performed on an Illumina MiSeq platform using primers targeting the V4-V5 region of the 16s rRNA gene. Data were analyzed using QIIME 1.8 Software (33-Caporaso et al. 2010). Alpha Diversity analyses revealed that the HF groups containing 5% grapes or 5% sugar resulted in reduced observed species compared to 3% grapes ($p = 0.0002$) or 3% sugar ($p < 0.0001$), respectively (**Fig 3.7**). Notably, HF diet containing 3% sugar reduced observed species compared to 3% grapes ($p=0.0026$), but there was no difference in observed species in 5% grape and sugar groups. These results suggest that elevated sugar content may result in decreased alpha diversity or membership of the gut microbiota. Principal Coordinate Analysis (PCoA) of weighted UniFrac distances did not reveal obvious differences in beta diversity or community structure across diet groups but there was clear separation between the LF group and HF groups based on unweighted UniFrac distances (**Fig. 3.8)**. To further interrogate differences between diet groups, multivariate statistical tests including

ADONIS, ANOSIM, and PERMANOVA were conducted. Significant differences were found among both unweighted and weighted UniFrac distances across diet groups (**Table 3.3**). Subsequently, OTUs that were not represented in up to 50% of the samples were removed to reduce noise from low abundance OTUs. Next, a Kruskal Wallis test was performed to determine significant differences in relative abundance of taxa between groups. Here, it was found that several bacterial taxa were significantly altered based on diet (**Fig. 3.9)**. While many genera were reduced in the HF diets compared to the LF diet, some were selectively increased in HF-3G group such as *Ruminoccocus* and *Anaeroplasma* in the Firmicutes and Tenericutes phylum, respectively (Fig. 3.9).

To assess differences between specific diet groups, Principal Component Analysis (PCA) was conducted and clear clustering was evident between the LF and HF-3G group (**Fig. 3.10A**) and between the HF-3G and HF-3S group (**Fig 3.10B**). Principal Component Analysis between the LF and HF-3G group revealed that ~60% of the variance in relative bacterial abundance between the two groups is explained with Principal Component 1 (PC1). PC1 exhibits strong correlations with two strains of bacteria: S24-7 within the Bacteroides phylum and *Clostridiales* in the Firmicutes phylum (Fig. 3.10A). This indicates that the variance explained by PC1 is primarily due to the relative abundances of these two bacterial strains. Principal component 2 (PC2) explained an additional 20% of the variance between groups and was primarily correlated with *Akkermansia*. PCA between the HF-3G and HF-3S groups revealed that ~60% of the variance in relative microbial abundance between the two groups was explained by PC1. PC1 was once again strongly correlated with S24-7 and *Clostridiales* (Fig.

3.10B). PC2 explained ~20% of the variance and exhibited a strong correlation with *Akkermansia*, however this was positively associated with the HF-3S group. These data suggest that most variance between the LF and HF-3G was explained by the abundance of S24-7 in the LF group. Interestingly, S24-7 also explained the variance between the HF-3G and HF-3S groups, as it was positively correlated with HF-3G compared to HF-3S. These data may suggest that S24-7 is associated with improved metabolic profile of the LF and HF-3G groups.

Lastly, Spearman Correlation analysis revealed significant negative correlations between body fat percentage and several genera (**Fig. 3.11**, **Table 3.4**) with the most profound being *Bifidobacterium* ($p = 0.0001$), a butyrate producer, and others including *Lactobacillus* ($p = 0.0221$) and *Allobaculum* ($p = 0.034$; Fig 3.11, Table 3.4). These particular genera were similarly correlated with inguinal fat pad weight. *Clostridiales* of the Firmicutes phylum was positively correlated with body fat percentage ($p = 0.0065$). Interestingly, a heatmap made from the taxa that were significantly correlated with body fat percentage, reveals that *Allobaculum* belonging to the Tenericutes phylum was increased in both the LF and HF3G group (**Fig. 3.11**). Little is known regarding the functional role of *Allobaculum* in the gut ecosystem and is currently being investigated for its potential association with a lean phenotype and improved metabolic health.

Discussion

Our data demonstrate that consuming table grapes (i.e., 3-5%, w/w; equivalent to 9-15 human servings) attenuates the accumulation of body and liver fat in mice fed a diet rich

in butter compared to control mice. However, these lipid-lowering effects of grapes were not associated with improvements in glucose tolerance or markers of inflammation in intestinal mucosa or WAT. Notably, the impaired localization of the intestinal tight junction protein ZO-1 in high fat-fed mice was improved by grape consumption. Populations of the deleterious sulfidogenic bacteria *Desulfobacter* spp, and the *Bilophila wadsworthia*-specific dissimilatory sulfite reductase gene were decreased by grape consumption (HF-3G), and populations of the beneficial bacterium *Akkermansia muciniphila* tended to be higher the colonic mucosa (HF-5G) or digesta (HF-3G) of grape fed mice compared to their respective controls. Additionally, via 16s rRNA sequencing analysis, we found significant differences across groups in the relative abundance of S24-7 and *Akkermansia* which were found to be negatively correlated with HF diet compared to the LF diet. Intriguingly, correlation analysis showed a strong negative correlation between *Bifidobacterium* and body fat percentage and inguinal fat pad weight. Taken together, these data indicate that consumption of table grapes attenuates adiposity and steatosis that are positively correlated with a marker of intestinal integrity and changes in several species of gut microbes in mice fed a butter-rich diet.

Influence of dietary polyphenols on adiposity, steatosis, and glucose tolerance

Many studies have demonstrated that consuming diets rich in calories from fat, particularly saturated fat, and sugars promote obesity and its metabolic complications [reviewed in 11]. The ability for foods rich in polyphenols, including grapes, to prevent obesity-mediated inflammation or related disorders has been demonstrated [reviewed in

25]. For example, grape seed procyanidin extract supplementation reduces body weight gain and adipose tissue mass in hamsters fed a HF diet [38]. Additionally, mice fed a HF diet supplemented with muscadine grape phytochemicals rich in anthocyanins had decreased body weights, less lipid accumulation in the liver, and improved glucose tolerance compared to high fat controls [39].

While several studies demonstrate anti-inflammatory and anti-obesity effects of grapes or their extracts, the mechanisms by which these effects occur are less clear. Anthocyanins are one of the most abundant phytochemicals in grapes [30] and therefore may be responsible for mediating the reductions in adiposity and steatosis. For example, anthocyanins purified from purple sweet potatoes attenuated hepatic lipid accumulation via the activation of adenosine monophosphate-activated protein kinase and decreased expression of SREBP1 and its downstream target genes in mice fed a HF diet [40]. Our study found that powdered grape supplementation at 3% and 5% reduced body weight and fat gain and hepatic lipid accumulation, but had no significant effects on the expression of *Srebp1c* and *Fas*. One explanation for the differential effects of extracted anthocyanins cited above [40] and our anthocyanin-rich grapes may be due to the binding of anthocyanins to fibers or proteins in the intact grapes, making them less bioavailable to the host. Although we discovered that the expression of several hepatic genes associated with lipogenesis (e.g., *Pparγ, Scd1, Fabp4, Gpat1*) in liver were lower in mice consuming the HF-3G compared to the HF-3S controls, the protein levels of associated with these genes were not lowered by grape feeding. However, we did not measure the

activity of these proteins, and therefore do not know they were impacted by grape consumption.

Relationship between dietary fat, sulfite-reducing bacteria, barrier function, and inflammation

Devkota et al. [10] demonstrated that feeding a milk-fat-based diet similar in amount and composition to the western diet (i.e., 37% kcals from fat) to IL-10 knockout mice decreased gut barrier-protecting bacteria and increased sulfate-reducing bacteria and increased intestinal markers of inflammation consistent with inflammatory bowel disease [10]. Mice consuming the milk fat diet had increased abundance of *B. wadsworthia*, which was associated with a pro-inflammatory T helper type immune response and increased colitis. Notably, taurine-conjugated bile salts caused these inflammatory responses, in part, due to their high sulfur content, which stimulates sulfite-reducing bacteria like *B. wadsworthia.* In a parallel 3 week study, these authors fed C57BL/6J mice the same high fat diet [10]. Consistent with the IL-10 knockout mice, C57BL/6J mice fed the milk fat diet had an increased bloom of the sulfidogenic bacteria *B. wadsworthia* and the *B. wadsworthia*-specific dissimilatory sulfite reductase gene *dsrA-Bw*. Milk fat-fed C57/6J mice also had a higher abundance of Bacteriodetes and a lower abundance of Firmicutes compared to the low fat-fed mice [10]. However, milk fat-fed C57BL/6J mice did not present overt colitis as did the IL-10 knockout mice [10]. In the present study, we did not observe any increases in the expression of sulfidogenic bacteria-related genes in the high fat-fed controls compared to the LF fed mice, although

the HF-3G had lower expression levels of *dsrA-Bw* and *DSB* in the ileum mucosa compared to the HF-3S control group (Fig. 3.6A). This decreased abundance of sulfidogenic bacteria correlated with improved localization of the ileal tight junction protein ZO-1 (Fig. 3.5). Although no markers of inflammation in the intestinal samples were significantly increased by the milk fat based diet or decreased by grape feeding, the expression levels of several inflammatory genes in visceral epididymal WAT (i.e., *Tnfα, Cd11c, Mcp1*) and subcutaneous inguinal WAT (i.e., *Mcp1*) were increased by HF feeding (Fig. 3.4A), and HF-5G consumption reduced *Mcp1*expression in inguinal WAT compared to its control HF-5S (Fig. 3.4A).

These results are comparable to data showing that HF feeding selectively increases the abundance of a specific type of bacteria that are associated with intestinal inflammation [41]. Apoa-I knockout mice, which present impaired glucose tolerance and increased adiposity, and wild type mice were fed a very HF diet (i.e., ~60% kcals from lard) for 25 weeks. HF fed mice had increased abundance of *Desulfovibrionaceae*, a family of sulfate/sulfite reducing bacteria that produce hydrogen sulfide, a genotoxic gas that causes barrier dysfunction and endotoxemia [42]. Consistent with these data, we showed that C57BL/6J mice fed a very HF diet (i.e., ~60% kcals from lard) for 20 weeks increased three types of sulfidogenic bacteria in colonic mucosa, impaired localization of ZO-1, and mRNA levels of markers of macrophage infiltration in intestinal mucosa and WAT compared to LF fed mice [32]. Collectively, these data demonstrate that consuming diets enriched with lard or butter, two fat sources containing high levels of saturated fatty

acids, increases the abundance or markers of sulfate-reducing bacteria associated with impairment of intestinal barrier function and contribute to systemic inflammation.

Relationship between dietary fat, Akkermansia muciniphila, barrier function, and inflammation

Akkermansia muciniphila, a commensal, mucin-degrading bacteria, plays a role in preventing the development of diet-induced obesity [37]. Under normal conditions, these bacteria represent 3-5% of the gut microbial population in humans. However, HF feeding reduces the colonic abundance of these bacteria in mice 100-fold, and oligofructose prebiotic treatment prevents this loss. Oligofructose-mediated increase in *A. muciniphila* was linked to decreased metabolic endotoxemia and markers of inflammation in WAT. Furthermore, supplementation of C57BL/6J mice fed a HF diet with *A. muciniphila* for 4 weeks lead to a decrease in adiposity, gut barrier dysfunction, metabolic endotoxemia, glucose intolerance, insulin resistance, and *Cd11c* expression in WAT, and improved mucus thickness along the epithelium compared to control mice [37]. Consistent with these data demonstrating that oligofructose prebiotic treatment attenuates a HF diet-induced reduction of *A. muciniphila*, our data indicate that supplementing high fat, butter-rich diets with 5% California table grapes increases the abundance of *A. muciniphila* in proximal colon mucosa compared to the HF-sugar controls (Fig. 3.6B). This increase in *A. muciniphila* was positively associated with improved localization of ZO-1 in the apical area of the ileal epithelium compared to HF control mice (Fig. 3.5). Furthermore, based on our 16s rRNA sequencing data of the cecum mucosa, the genus *Akkermansia* was

positively associated with mice fed a LF diet, further supporting a relationship with a lean phenotype.

Limitations and unanswered questions

The current study did not achieve the anticipated increases in intestinal and systemic inflammation in young C57Bl/6J mice fed a high fat diet rich in milk-fat as demonstrated in IL-10 knockout mice fed milk-fat [10] or in Apoa-I knockout mice fed lard [41]. Perhaps feeding mice extremely HF diets (e.g., 60% kcals from lard) is necessary to instigate intestinal and systemic inflammation [11, 30]. Our current study sought to provide a more physiological approach to achieve diet-induced obesity accompanied by systemic and intestinal inflammation. The use of this more physiological model for the time span of 11 weeks may not have been aggressive enough in this model to elicit a robust inflammatory response, as the percentage of calories from fat (34% of kcals) or the composition of fat (primarily milk fat) may not have been adequate, to induce a severe case of obesity and inflammation. Future studies should examine the effect of different polyphenols or fractions present in grapes on obesity and systemic and intestinal inflammation using a diet induced obesity mouse model fed a diet that is more representative of the fat composition of the typical American diet (i.e., a mixture of animal and vegetable fats consisting of beef tallow, lard, milk fat, shortening, and vegetable oils [43].)

Acknowledgements

We would like to acknowledge the contributions of everyone involved in collecting data for this study. Brian Collins and Jessie Baldwin equally conducted all stages of the animal study, including caring, feeding, and weighing of the mice, the glucose tolerance testing, and the measurement of body fat using DEXA. Brian Collins additionally measured markers of ileal and colonic gene and protein expression associated with inflammation, ileal activity of myeloperoxidase, and duodenal activity alkaline phosphatase. Jessie Baldwin additionally measured markers of inguinal and epididymal gene and protein expression associated with inflammation, fat depot and liver gene expression of markers of lipolysis and lipogenesis, and serum triglyceride levels. Robin Hopkins measured plasma insulin using an ELISA assay. Chia-Chi Chuang measured liver triglyceride levels. H.R. Gaskins and Patricia Wolfe together measured the PCR of 16s rRNA for sulfidogenic bacteria and *A. muciniphalia* markers. Kristina Martinez, Eugene Chang measured the sequencing of 16s rRNA using the Illumina Mi-seq platform and Chase Cockrell conducted the analyses of data shown in Figures 3.7-3.10. Wei Zhong measured the ileal localization of the tight junction protein ZO-1. Paula Cooney measured the body fat mass using DEXA (with the assistance of Brian Collins and Jessie Baldwin).

Table 3.1. Diet Formulations

Ingredients (gram)	LF	HF-3S	HF-3G	HF-5S	HF-5G
Casein	200	200	200	200	200
L-Cystine	3	3	3	3	3
Corn Starch	506.2	194	194	194	194
Maltodextrin 10	125	94	94	94	94
Fructose	0	13.3	0	21.9	21.9
Dextrose, Monohydrate	0	13.3	0	21.9	21.9
Sucrose	68.8	154	154	139	139
Cellulose	50	50	50	50	50
Soybean Oil	25	12	12	12	12
Butter	0	144	144	144	144
Lard	20	0	0	0	0
Mineral Mix	10	10	10	10	10
DiCalcium Phosphate	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5
Potassium Citrate	16.5	16.5	16.5	16.5	16.5
Vitamin Mix	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2
Grape Powder	0	0	28.5	0	47
Total	1055.1	854.7	857.5	855.7	857.7

LF, low fat control; HF-3S, high fat- 3% sugar control; HF-3G, high fat- 3% grapes; HF-5S, high fat-5% sugar control; HF-3G, high fat-5% grapes

Table 3.2. The Effect of Diet Composition on Weight Gain, Consumption, and Utilization.

Diets	BWG n=9-10	FI n=5	FCE n=5	Kcal n=5
LF	7.0 ± 0.6^{b}	399 ± 8^{b}	28.6 ± 1.2^{a}	1534 ± 26^{b}
HF-3G	13.1 ± 0.7^{a}	465 ± 7^{a}	17.8 ± 0.8^{b}	2051 ± 26^{a}
HF-3S	11.5 ± 0.7^{a}	424 ± 20^{ab}	18.7 ± 0.9^{b}	1874 ± 80^{a}
HF-5G	11.3 ± 1.1^{a}	409 ± 12^{ab}	18.4 ± 1.1^{b}	1799 ± 45^{ab}
HF-5S	12.6 ± 1.0^{a}	438 ± 25^{ab}	17.8 ± 1.5^{b}	1957 ± 124^{a}

Mean ± SEM without a common lower case letter in a column differ ($p<0.050$ using one-way ANOVA and Student's t test. BWG = total body weight gain (g). FI= total food intake per cage (g). FCE= food conversion efficiency per cage. Kcal= total caloric intake per cage (kcals).

Table 3.3. Statistical Analyses of Unifrac Distances

Weighted	**R2**	**P Value**
Adonis	0.12361	0.001
Anosim	0.3048	0.01
	Pseudo F Statistic	**p value**
Permanova	1.4105	0.001
Unweighted	**R2**	**P Value**
Adonis	0.23825	0.001
Anosim	0.2609	0.01
	Pseudo F Statistic	**p value**
Permanova	3.1277	0.001

Table 3.4. Taxa Associated with Body Fat

Taxa Associated with Body Fat Percentage: (Phylum; Class; Order; Family; Genus; Species)	**Correlation**	**P Value**
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	-0.53	0.0001
Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	-0.33	0.0221
Firmicutes; Clostridia; Clostridiales; Other; Other	0.39	0.0063
Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Other	-0.32	0.0243
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus	-0.35	0.0147
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum	-0.30	0.0369
Taxa Associated with Inguinal Fat Pad Weight: (Phylum; Class; Order; Family; Genus; Species)	**Correlation**	**P Value**
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	-0.48	0.0004
Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	-0.39	0.0053
Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Leuconostoc	-0.29	0.0413
Firmicutes; Clostridia; Clostridiales; Other; Other	0.38	0.0065
Firmicutes; Clostridia; Clostridiales; Clostridiaceae	-0.34	0.0177
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Ruminococcus	-0.35	0.0131
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum	-0.30	0.0391
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Coprobacillus	0.31	0.0297
Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma	-0.30	0.0380

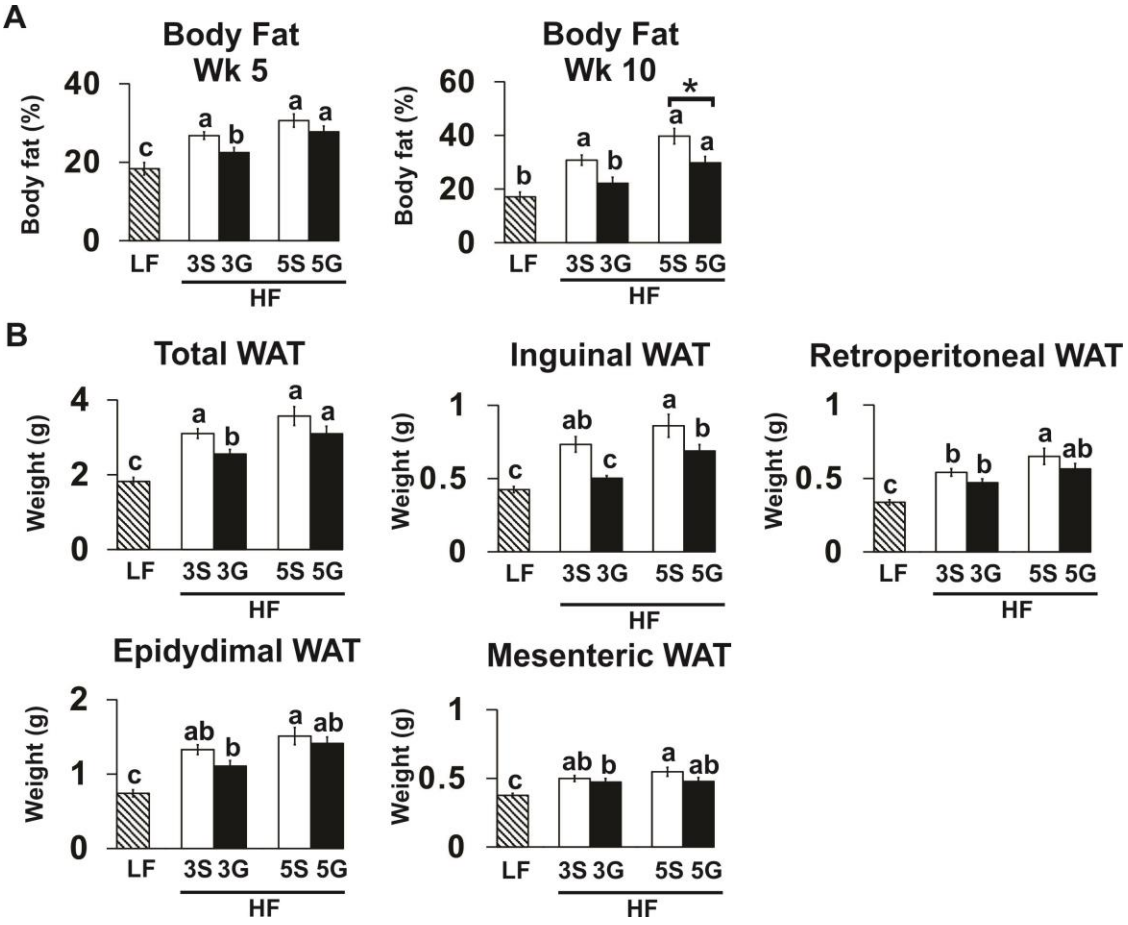

Figure 3.1. Diet Induced Changes to Fat Mass. Adiposity indices of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Body fat percentages were measured at week 5 and week 10 using dual energy x-ray absorptiometry (DEXA). (B) At week 11, epididymal, inguinal, retroperitoneal, and mesenteric white adipose tissue (WAT) depots were excised and weighed (B). The weights of the epididymal, inguinal, retroperitoneal, and mesenteric depots were measured, and their sum labelled total WAT. Means ± SEM without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's t test. Means ± SEM (n=9-10) sharing the symbol "*" differ using the Bonferroni's adjustment ($p<0.01$).; LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

Figure 3.2. Glucose Tolerance Tests. Glucose tolerance tests (GTT) s of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. At weeks 3, 6, and 9, GTTs were conducted on mice fasted for 8 h and injected i.p. with a 20% glucose solution. Data are expressed as total area under the curve (AUC) for the GTTs. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

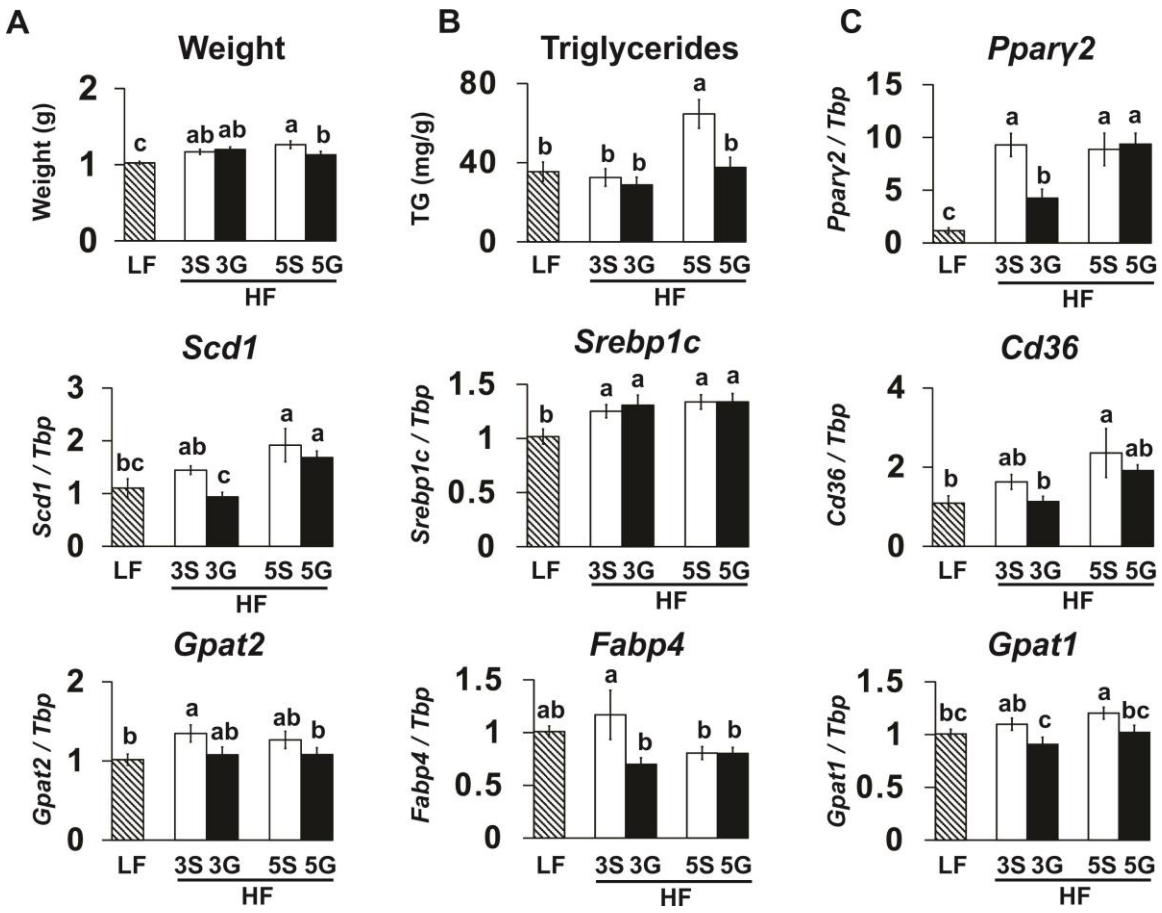

Figure 3.3. Liver Gene Expression. (A) Liver weights, (B) liver triglyceride levels, and (C) the expression of several lipogenic genes in liver of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. qPCR was conducted to measure mRNA abundance of genes associated with hepatic lipogenesis. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

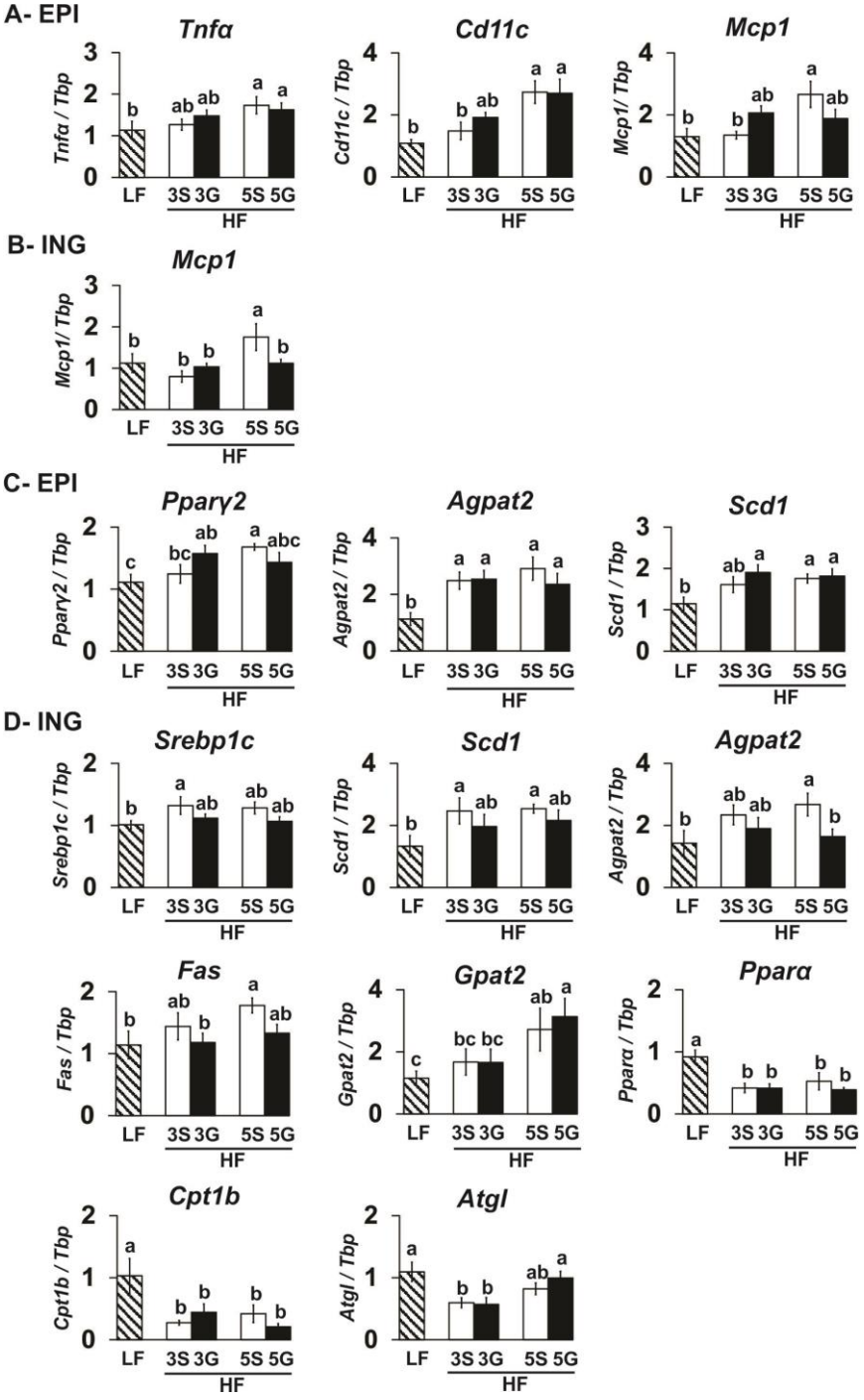

Figure 3.4. WAT Gene Expression. The expression of markers of inflammation and lipid metabolism in epididymal and inguinal WAT of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks.

qPCR was conducted to measure mRNA abundance of genes associated with inflammation in (A) epididymal (EPI; visceral) and (B) inguinal (ING; subcutaneous) WAT depots and also mRNA abundance of genes associated with lipid metabolism in (C) epididymal (EPI; visceral) and (D) inguinal (ING; subcutaneous) WAT depots. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

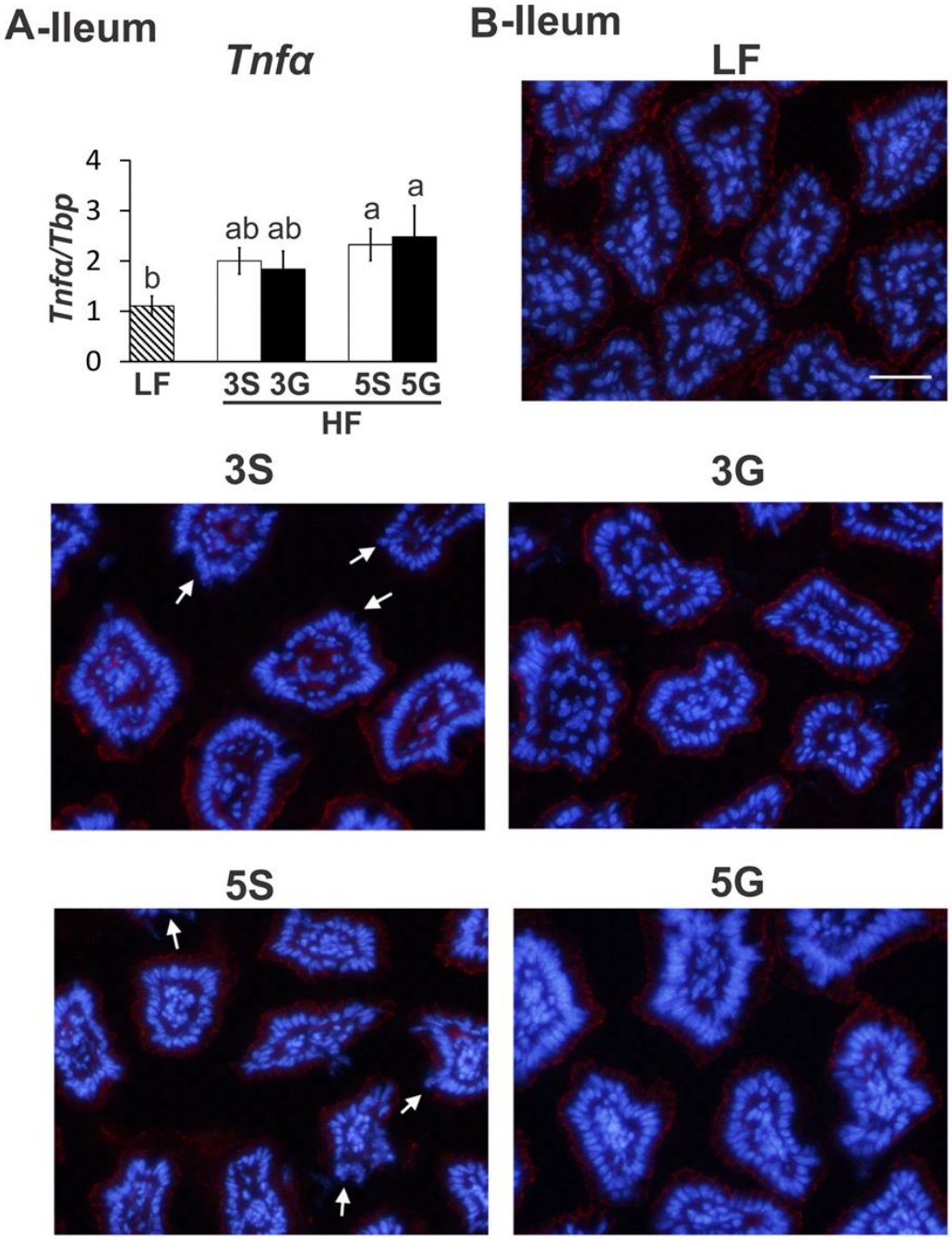

Figure 3.5. Alterations to Intestinal Inflammation and Barrier Function. (A) Ileum mucosa expression of TNFα and (B) the localization of the tight junction protein ZO-1 at the apical area of the ileum epithelium in C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. qPCR was conducted to measure mRNA abundance of inflammatory genes. Localization of ZO-1

was visualized by immunostaining of ileum samples (n=5). Means ± SEM (n=9-10 for TNFα) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

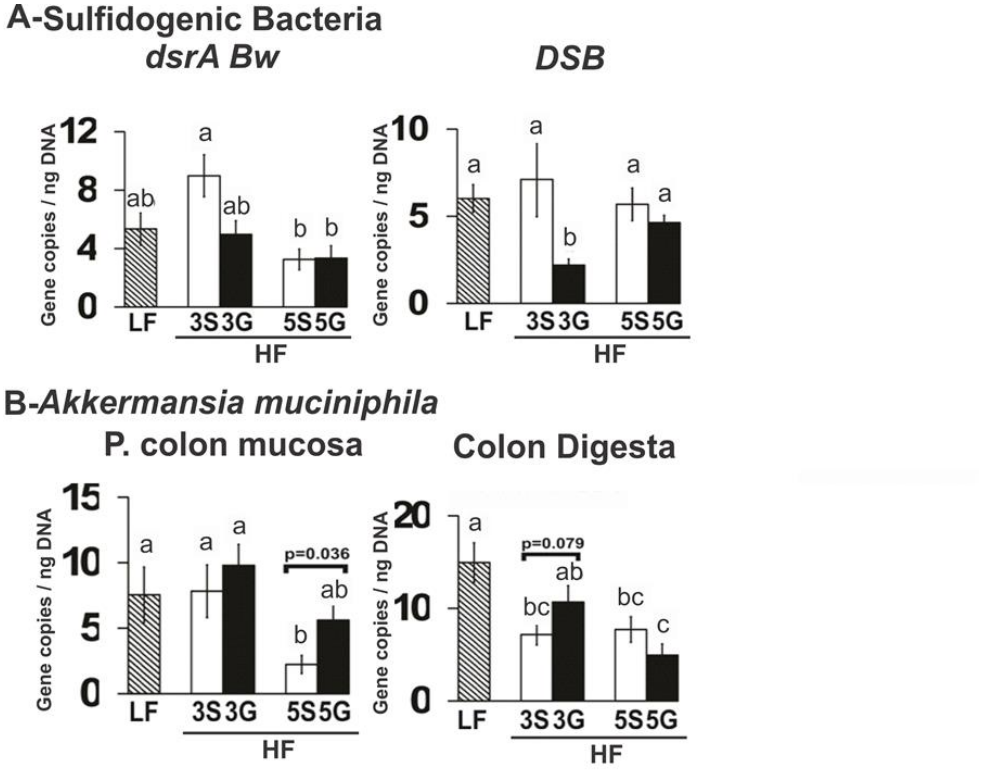

Figure 3.6. Diet Induced Alterations to Sulfidogenic Bacteria and *Akkermansia muciniphilia*. Abundance of sulfidogenic bacteria and *Akkermansia muciniphilia* in the intestinal mucosa or digesta of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Abundance of the sulfidogenic, *Bilophila wadsworthia*-specific, functional gene target dissimilatory sulfate reductase (*dsrA-Bw*) and the targeted sulfidogenic bacterial genera *Desulfobacter* (DSB) species in the ileum mucosa. (B) The abundance of the probiotic *Akkermansia muciniphilia* in colon mucosa and digesta and in cecum digesta. qPCR was conducted to measure mRNA abundance of the functional genes and bacterial genera. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$).

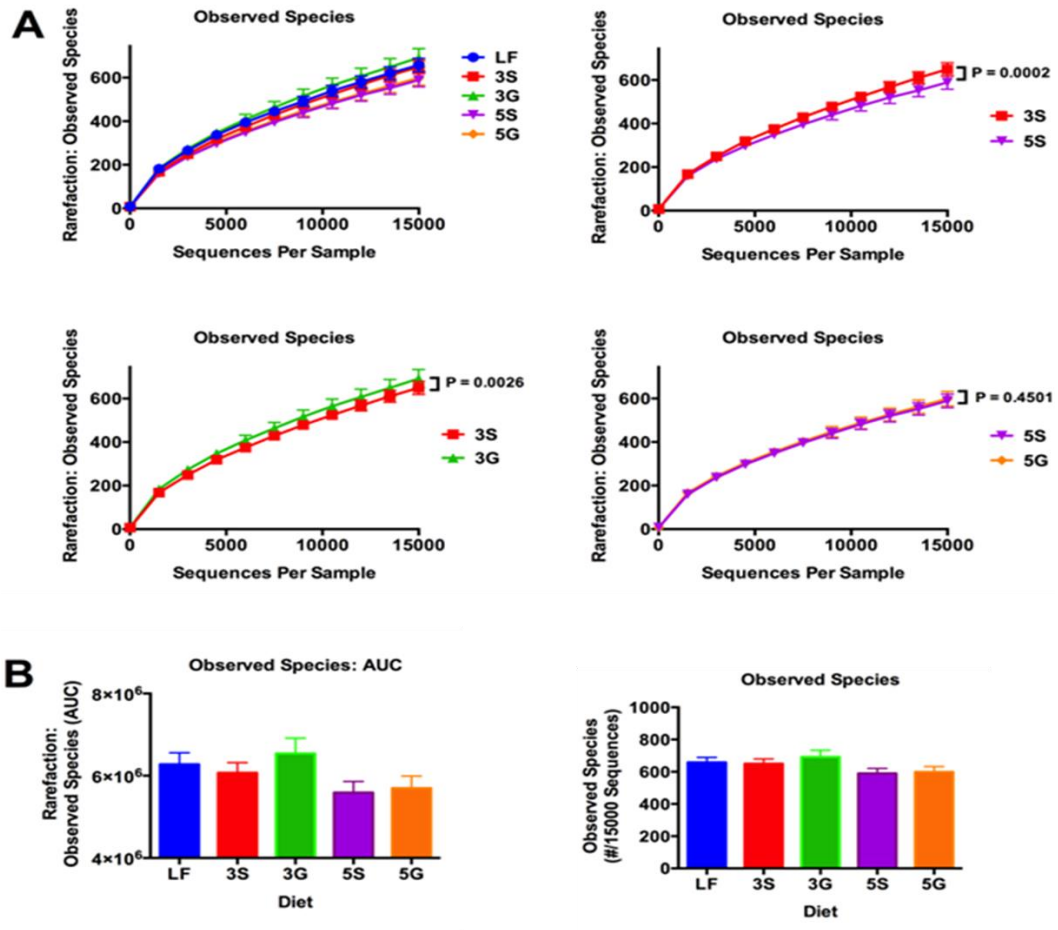

Figure 3.7. Diet Induced Changes to Microbial Species. Observed bacterial species in cecum mucosa of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Rarefaction curves of observed species are shown. Samples were rarified to 15,000 sequencing reads per sample. (B) Above - Area Under the Curve (AUC) of rarefaction curves shown for each diet group. Below – Observed species at 15,000 sequencing reads for each diet group. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes. Data are presented as Means ± SEM (n=9-10) using one-way ANOVA and Dunn's test for multiple comparisons.

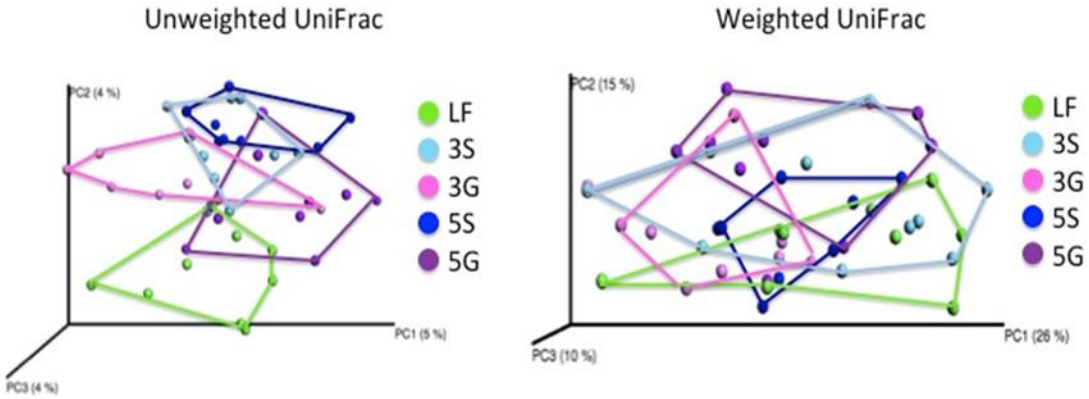

Figure 3.8. PCoA Plots of Unweighted and Weighted UniFrac Distances. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

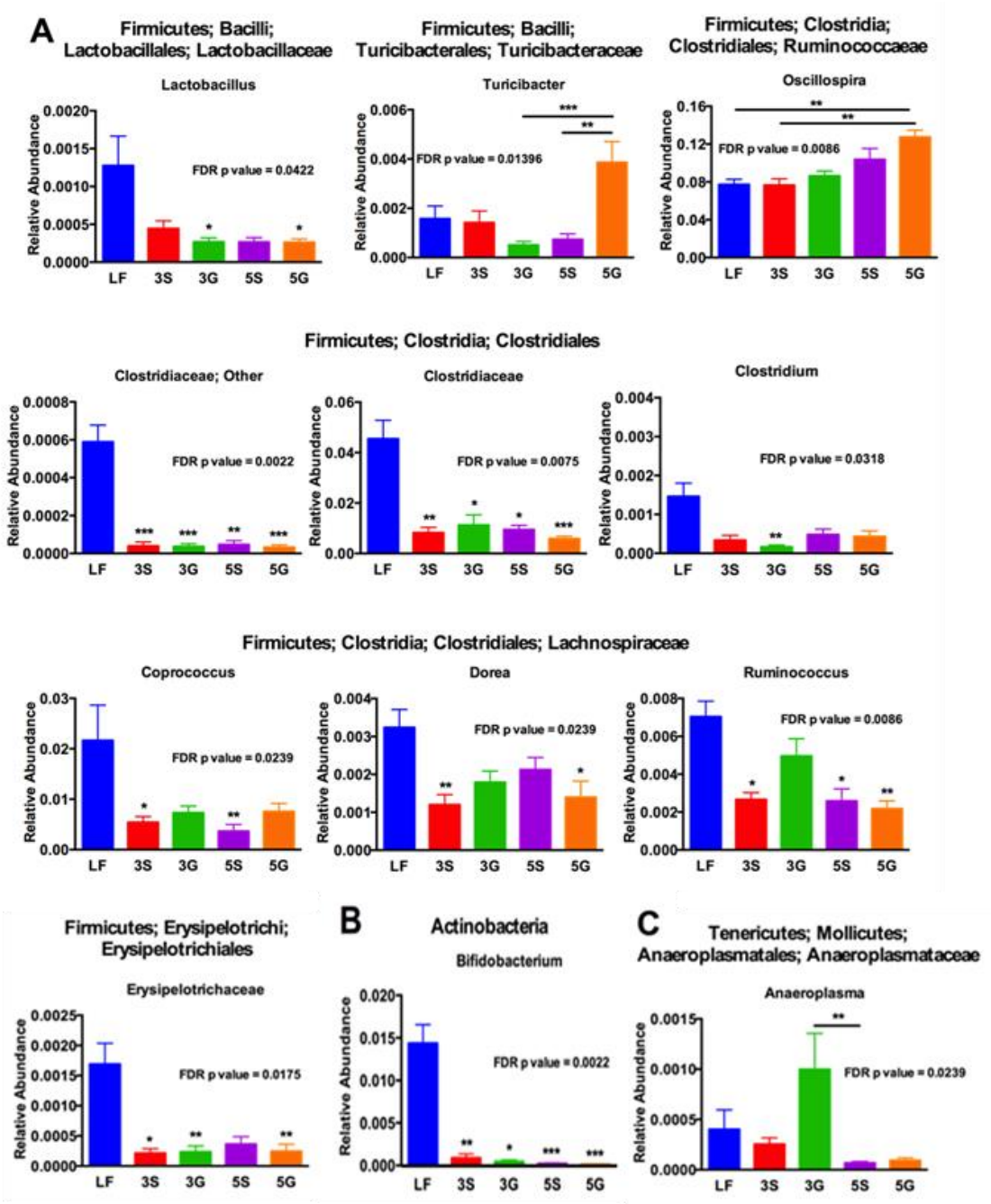

Figure 3.9. Relative Abundance of Microbial Taxa. Significantly altered relative abundances of microbial taxa (i.e., A- Firmicutes, B- Actinobacteria, and C- Tenericutes) found across C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. Taxa shown were significantly altered

based on Kruskal Wallis test run using QIIME software following filtering of OTUs that were not present in 50% of samples. FDR corrected p values based on this analysis are shown. To conduct multiple comparisons relative abundances were analyzed via ANOVA followed by Dunn's test for multiple comparisons. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes. Data are presented as Means ± SEM (n=9-10). Unless otherwise indicated, asterisks show significant differences of HF diets with or without grapes compared to LF control.

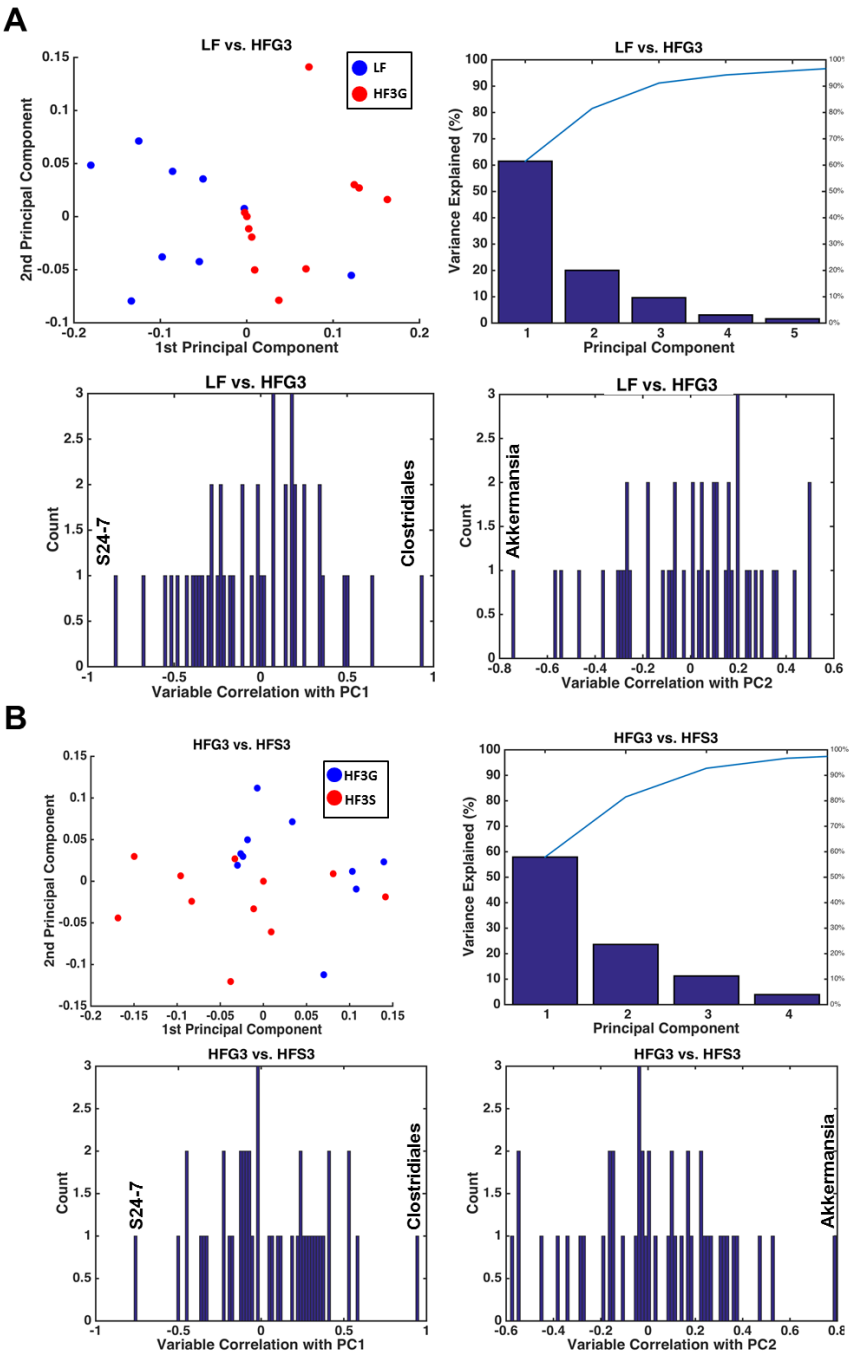

Figure 3.10. Principle Component Analysis (PCA) of Cecum Mucosa. The microbial relative abundances from C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Left - PCA plot between low fat (LF) and high fat + 3% grapes (HF3G) are shown. Middle – Pareto plot showing

percentage of variance explained by principal components. Right – Histogram showing individual genera that were negatively or positively correlated with LF and/or HF-3G diets. (B) Same as A but showing PCA of HF-3G vs high fat + 3% sugar (HF-3S) groups.

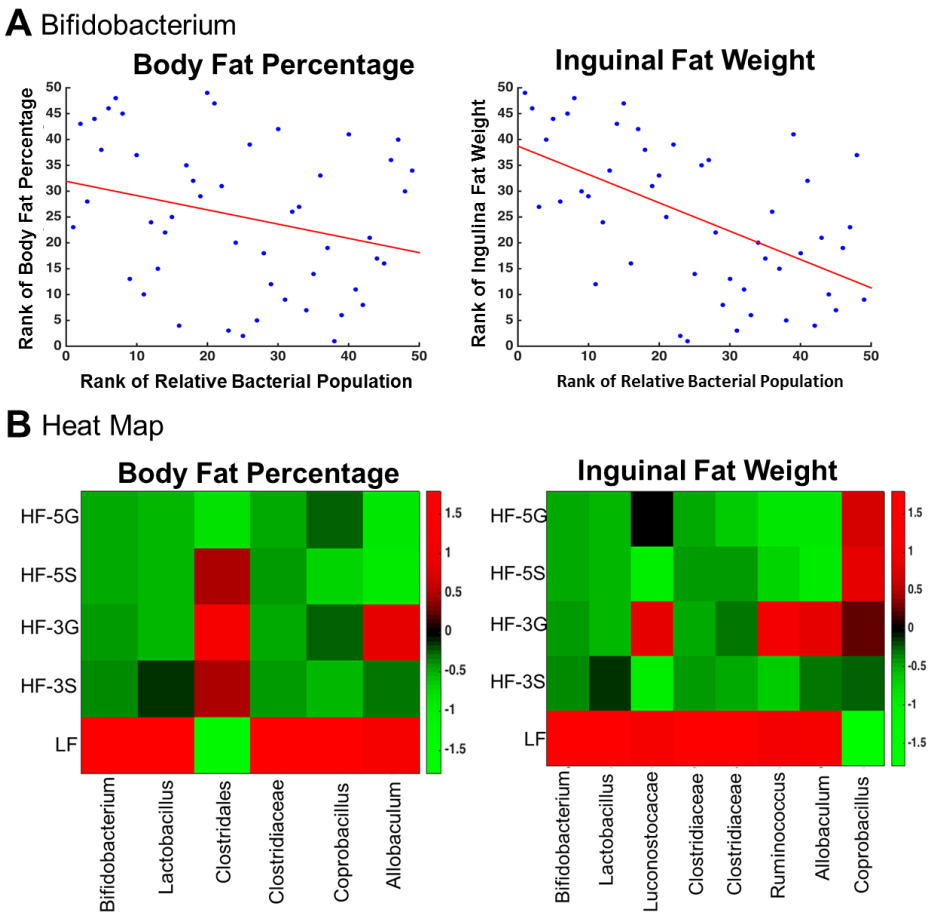

Figure 3.11. Relationship between Body Fat and Bacterial Populations. (A) *Bifidobacterium* relative abundance is negatively correlated with body fat percentage (r = -0.53, p = 0.001) and inguinal fat pad weight (r = -0.48, p = 0.004) as determined by Spearman Correlation Analysis. (B) Heatmap showing relative abundances of taxa that were significantly correlated with body fat percentage. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

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CHAPTER IV

AN EXTRACTABLE, POLYPHENOL-RICH FRACTION OBTAINED FROM CALIFORNIA TABLE GRAPES DECREASES ADIPOSITY, INSULIN RESISTANCE, AND MARKERS OF INFLAMMATION IN HIGH-FAT FED C57BL/6J

Jessie Baldwin, Brian Collins, Chia-Chi Chuang, Paula Cooney, Robin Hopkins, and Michael McIntosh

Abstract

The objective of this study was to determine the extent to which consuming two methanol-extractable fractions of California table grapes reduced adiposity, hepatic steatosis, or markers of inflammation or lipid metabolism in mice fed a high-fat, American-type diet. Male C57BL/6J mice were fed a low fat diet, a high fat (HF) diet, or a HF diet containing California table grapes (GP; 5% w/w), an extractable polyphenol (HF-EP) fraction from GP, a non-extractable polyphenol (HF-NEP) fraction from GP, or an equal combination of both fractions (HF-EP+NEP) for 16 weeks. Mice fed the HF-EP and HF-EP+NEP-containing diets had lower percentages of body fat and amounts of total white adipose tissue (WAT) and improved glucose tolerance compared to the HF controls. In epididymal WAT, the mRNA levels of the inflammatory genes cluster of differentiation 11c, monocyte chemoattractant protein 1, epidermal growth factor-like module-containing mucin-like hormone receptor 1, tumor necrosis factor alpha, and the lipogenic gene, acylglycerol-3-phosphate-O-acyltransferase *2* were lower in mice fed the

HF-EP- and HF-EP+NEP-containing diets compared to HF controls. Mice fed HF-NEP and HF-EP+NEP diets had reduced liver weights and mice fed HF-EP+NEP diets hadlower liver triglyceride levels compared to the HF controls. Mice fed the HF-EP+NEP diets had higher hepatic mRNA levels of hormone sensitive lipase and adipose triglyceride lipase, and decreased expression of c-reactive protein compared to the HF controls. Surprising, HF-GP feeding neither decreased adiposity, hepatic steatosis, or inflammation nor did the HF or grape diets alter markers of intestinal inflammation. Taken together, these data demonstrate that the polyphenol-rich EP fraction from California table grapes attenuates many of the adverse health consequences associated with consuming a HF diet, independent of influencing intestinal inflammatory status.

Introduction

Obesity has been on the rise in the U.S. since the 1960's and affects approximately one third of the adult population [1]. The incidence of obesity is also increasing globally, affecting approximately 300 million adults worldwide [2]. Obesity is intimately associated with chronic inflammatory conditions that contribute to the metabolic syndrome (e.g., type 2 diabetes, hypertension, and cardiovascular disease) [3]. Over consumption of calories coinciding with a lack of physical activity are the major risk factors for obesity development resulting in expansion of white adipose tissue (WAT). As WAT grows beyond the capacity of the vasculature system to provide it with nutrients and oxygen, inflammatory signals recruit macrophages and other immune cells into the WAT. As the immune response progresses, the abundance of circulating and tissue

proinflammatory cytokines and chemokines increases [4]. This inflammatory scenario disrupts metabolic processes which results in impaired glucose and fatty acid uptake and metabolism, and hemostasis, thereby contributing to the development of metabolic diseases. Although there is a general consensus on the metabolic consequences of obesity-mediated inflammation, the exact mechanisms that initiate these events are not clearly understood.

Recently, the role that gut microbes play in the development of the metabolic syndrome has received attention due to their sensitivity to environmental changes that can trigger obesity [5], chronic inflammation [6-8], and insulin resistance [9]. Diets high in fat have been implicated in the reduction of microbial diversity, in particular gut barrier-protecting bacteria as well as increasing the abundance of deleterious bacteria [reviewed in 10]. For example, an increase in the ratio of *Firmicutes* to *Bacteriodetes* is positively correlated with the development of obesity and insulin resistance [11]. Diets rich in saturated fat [11, 12], particularly from milk [13], increase the abundance of sulfidogenic bacteria like *Bilophila wadsworthia* and *Desulfovibrionaceae* spp. which generate hydrogen sulfide gas. Hydrogen sulfide is geno- and cytotoxic and positively correlated with development of ulcerative colitis, gut inflammation, irritable bowel syndrome, and colon cancer [13-15]. Notably, the effects of high fat (HF) diet on body weight gain are repressed in microbiota-free mouse models as well as fecal microbial transplants from healthy donors into obese subjects [16]. Therefore, interventions directed towards modifying the microbial composition of the gastrointestinal (GI) tract may alleviate obesity-mediated outcomes.

The use of non-digestible carbohydrates, fiber, or polyphenols as prebiotics shows promise as potential interventions for the metabolic consequences of obesity [reviewed in 13, 17-19]. Prebiotics are agents that selectively stimulate the growth or activities of specific microbial populations in the gut which translates into health benefits for the host [20]. Fiber, in particular inulin-type fructans, has been shown to increase the abundance of *Bifidobacteria* which was positively correlated with decreased hyperglycemia, endotoxemia, and systemic cytokine levels [21, 22]. Similar effects have been demonstrated in obese subjects with short term supplementation of gluco-oligosaccharides [23]. It is believed that the primary benefit of fiber fermentation by intestinal microbes is through increased productions of short chain fatty acids (SCFA), which have be shown to regulate the synthesis of GI peptides that influence energy intake (i.e., glucagon-like peptide (Glp)-1 and 2, peptide YY (Pyy), and ghrelin) as well as energy storage and metabolism through interactions with G-protein receptors (Gpr) 41 and 43 [reviewed in 20].

Although much focus has been placed on the beneficial properties of fiber, polyphenols found in fruits and vegetables may also improve GI health [24-27]. Absorption of polyphenols is poor in the upper gastrointestinal tract, leading to increased availability in the lower GI tract [27]. Thus, polyphenols may have a significant influence on microbiota taxa and their metabolites [28]. Indeed, the anti-inflammatory, anti-oxidant, and anti-microbial actions of polyphenols have been reported to positively influence gut microbes and host inflammation [reviewed in 29]. As such, one potential

mechanism to improve the outcomes associated with diet-induced obesity is increased consumption of poorly digestible food components including polyphenols.

Grapes and other berries are rich in polyphenols including anthocyanins [reviewed in 29], which have known anti-inflammatory and anti-oxidant effects [30]. These beneficial effects of grapes have been associated with reduced cytokine levels via suppression of nuclear factor kappa B (NFκB) and increased peroxisome proliferator-activated receptor (PPAR)α/γ [31]. Consistent with these data, we demonstrated that C57BL/6J mice consuming a HF diet (i.e., 60% kcals from lard) supplemented with whole powdered California table grapes (3%, w/w) had improved glucose tolerance after 5 weeks and decreased markers of inflammation ~20-50% in serum and WAT after 18 weeks [32]. We also demonstrated that consuming a moderate fat diet (i.e., 34% kcals from milk fat) supplemented with whole powdered grapes (3% or 5% w/w) reduced adiposity, improved liver triglyceride (TG) levels, modestly reduced WAT inflammatory gene expression, and lowered the cecum levels of sulfidogenic bacteria, while tending to increase the abundance of *Akkermansia muciniphalia* and *Allobaculum* in the proximal colon and cecum, respectively (Chapter III).

However, the identities of the bioactive fractions in whole powdered table grapes and the role that gut microbiota play in improvements in diet-induced obesity in mice fed grapes are unknown. Therefore, the objective of this study was to determine the extent to which consuming methanol-extractable fractions of table grapes reduced adiposity, hepatic steatosis, or markers of inflammation or lipid metabolism in mice fed a high-fat, American-type diet. We separated lyophilized powder from whole grapes into two

methanol-extractable fractions; i.e., an extractable polyphenol (EP) fraction and a non-extractable polyphenol (NEP) fraction. These fractions were incorporated into a HF, American-type diet (i.e., similar in fat amount and type to the 75th percentile of American diets [33]). This HF diet was fed alone or in combination with the EP fraction, the NEP fraction, both fractions (EP+NEP), or whole grape powder (GP; 5%, w/w) for 16 weeks. Body composition and glucose tolerance were measured at 5 week intervals. At week 16, markers of intestinal and systemic inflammation, insulin resistance, and lipid metabolism were measured.

Materials and Methods

Reagents and materials

Reference compounds procyanidin A2 (PAC-A2), procyanidin B2 (PAC-B2), catechin and epicatechin were purchased from Chromadex (Irvine, CA). 4-dimethylaminocinnamaldehyde (DMAC), Folin-Ciocalteu reagent, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). All organic solvents were HPLC grade and obtained from VWR International (Suwanee, GA).

Extraction and polyphenol enrichment

Freeze-dried grape powder (200 g x 5 batches) was blended each with 1 L 50% acidified methanol (0.1% TFA). The mixture was centrifuged (Sorvall RC-6 plus, Asheville, NC) at 4000 rpm for 10 min, and the supernatant was collected. A sample from the combined supernatants (2 L) was evaporated and freeze-dried to afford the

crude extract. The rest of aq-methnol extract was evaporated to remove the organic solvent, and then loaded to Amberlite XDA-7 resin, stirred for 20 minutes, and the supernatant was discarded. The resin was washed with water to get rid of all free sugars and organic acids. The polyphenols were eluted from the resin with 100% methanol, organic solvent was evaporated under vacuum, and the remaining aqueous extract was freeze-dried to afford the EP fraction. The pelleted material (plant debris after extraction) was put in a vacuum oven (45 ºC) to get rid of the organic solvent before freeze-drying to afford the NEP fraction.

Alkaline hydrolysis of NEP fraction

Alkaline hydrolysis of NEP fraction was performed according to Yang et al. [34] with some modifications. In 15 mL centrifuge tube, 2.0 mL of 4 M NaOH were added to 0.5 g NEP, flushed with nitrogen, closed and incubated for 1 hour at room temperature. The mixture was adjusted to pH 7 with drops of concentrated hydrochloric acid, than loaded onto a column packed with celite at a ratio 1:2 v/w. The hydrolyzed polyphenols were eluted with 30 mL methanol-ethyl acetate (20:80 v/v), and evaporated to dryness.

Determination of total phenolics, anthocyanins and proanthocyanidins

Total phenolics (TP) were determined in EP and NEP fraction hydrolysates with Folin-Ciocalteu reagent [35]. Concentrations were expressed as mg/L gallic acid equivalents. Total monomeric anthocyanin (ANC) content was measured in EP using the pH differential spectrophotometric method [36], and expressed as cyanidin glucoside equivalent. Total proanthocyanidin content (PAC) was determined in EP using the

DMAC method as previously described [37], and quantified as procyanidin A2 equivalent.

HPLC profile analyses of anthocyanins and proanthocyanidins

HPLC analyses were conducted according to the previously reported protocols [38].

Animals

Four-week old, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and acclimated on a standard chow diet for 1 week. Mice were housed in pairs, maintained at a temperature of 22°C with 50% humidity, and exposed to a 12 h light/ 12 h dark cycle. Mice received food and water *ad libitum* and measures of body weight and food intake were conducted once and twice per week, respectively. All experimental procedures were performed under ethical standards and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Greensboro.

Diets

Animals were randomly assigned to one of six dietary treatments (n=13 per treatment group) as follows: low fat (LF; 10% of energy from fat), HF (45% of energy from fat), HF plus extractable polyphenol fraction (HF-EP), HF plus non-extractable polyphenol fraction (HF-NEP), HF plus extractable and non-extractable polyphenol fraction (HF-

EP+NEP) and HF plus 5% powdered grapes (HF-GP) (**Table 4.1**) . The HF diets consisted of approximately 10.7% energy from soybean oil, 9.8% of energy from butter, 7.8% energy from lard, 12.2% energy from shortening, and 4.5% energy from beef tallow [33, 39]. Thus, the HF diets were rich in fat with the proportion of the sources of fat mimicking an American-type diet [33]. The amounts of EP and NEP fractions that were added to the HF diets were equal to their relatively amounts in the in the 5% powdered whole grape diet (i.e., grape powder contains 2.3% and 6.9% EP and NEP, respectively). The lyophilized (i.e., powdered) California table grapes were kindly provided by the California Table Grape Commission and consisted of a mixture of red, green, and purple seeded and seedless grapes. The 5% dietary level of grapes is comparable to 15 human servings of grapes. Because the extraction process removes the sugar fraction in grapes, a mixture of fructose and glucose was added to the HF, HF-EP, HF-NEP, and HF-EP+NEP diets to control for the sugar content of the powdered grape diet. Detailed composition of the diets is illustrated in Table 4.1.

Intraperitoneal glucose tolerance tests (GTT)s, and fasting insulin levels

Intraperitoneal (i.p.) GTTs were performed on weeks 7, 12, and 16 on non-anesthetized mice. Mice were fasted for 8 h and given an i.p. injection of glucose (i.e., 20% solution at 1 g/kg body weight). Blood from the tail vein collected at baseline and 5, 15, 30, 60, and 120 minutes post-i.p. glucose injection was used to quantify glucose levels using a Bayer Contour blood glucose monitor and strips (Bayer Healthcare, Tarrytown, NY, USA). Plasma insulin levels were detected using an ultrasensitive mouse

insulin kit (Crystal Chem, Inc, Downers Grove, IL). The homeostasis model assessment method (HOMA) for insulin resistance (IR) was used employing the following formula: [fasting insulin concentration (ng/ml) x 24 x fasting glucose concentration (mg/dl)] / 405 [32].

Body fat measurements via Dual X-Ray Absorptiometry (DEXA)

Percent body fat was measured using DEXA on a GE Lunar Prodigy Advanced System (GE Healthcare, Milwaukee, WI) at weeks 6, 11, and 15. During the measurement, mice were lightly anesthetized with isoflurane using a SomnoSuite Small Animal Anesthesia System with Integrated Digital Vaporizer isoflurane system. Measurements were taken in duplicate or triplicate to reduce the possibility of error and values expressed are an average of the two measurements.

Tissue collection

After 16 weeks of dietary intervention, mice were fasted for 8 h and euthanized via isoflurane-induced anesthesia followed by cardiac puncture. Plasma was collected at the time of harvest. Four WAT depots were collected; epididymal, mesenteric, inguinal, and retroperitoneal. Additionally, livers were harvested and intestinal mucosa and digesta were collected from the duodenum, jejunum, ileum, cecum, or proximal or distal colon. Weights of the WAT depots and liver were recorded and all collected samples were immediately snap-frozen in liquid nitrogen and stored at -80^{o}C until analysis.

Liver and plasma TG levels

Liver TG content was measured as previously described [40]. Plasma TG content was determined using a commercial assay from Thermo Scientific and was conducted following the manufacturer's protocol (Infinity TG assay #TR22421 and TG standards #TG22923; Norcross, GA).

Liver Oil-Red-O Staining

Liver tissues were frozen in OCT compounds, cut at 5 μm, and mounted on slides. The sections were fixed with 10% formalin for 10 minutes and then the slides were washed with deionized water for 5 minutes. Fixed tissues were then rinsed with 60% isopropanol for 5 minutes. To prepare Oil red O stock, 0.5 g of Oil red O (Sigma-Aldrich) was mixed with 100 mL of isopropanol. To prepare Oil red O working solution, 30 mL of Oil red O stock was mixed with 20 mL of distilled water and filtered using a 0.24 μm vacuum filter. Samples were submerged in the working solution for 15 minutes, briefly rinsed with 60% isopropanol, and then rinsed with deionized water for 30 seconds before imaging.

RNA extraction and qPCR

Adipose and intestinal samples were homogenized in QIAsol reagent and total RNA was extracted using QIAgen mini lipid kit obtained from Qiagen (Valencia, CA). For hepatic samples, a QIAgen mini universal kit from Qiagen was used. The quality and concentration of RNA were examined using absorbance at 260 nm and integrity determined using the absorbance ratio of 260/280 on a Nanodrop ND-1000

Spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA was created by reverse transcription using 1 ug of RNA and a high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocols. qPCR was performed in a 7500 FAST Real Time PCR system (Applied Biosystems). The expression of different genes related to inflammation, lipogenesis, and lipolysis in WAT depots and liver were measured using Taqman Gene expression assays purchased from Applied Biosystems. TATA-binding protein (*Tbp*) was the endogenous reference gene utilized for all assays and fold differences in gene expression were calculated as $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Data were analyzed using a one-way ANOVA and Student's *t* tests to compute individual pairwise comparisons of means ($p<0.05$). Bonferroni's posthoc tests were also performed for specific comparisons where appropriate. Analyses were conducted using the JMP software program version 10.0 for Windows (SAS, Cary, NC). Data are expressed as means + S.E.M.

Results

Polyphenol content and profile of EP, NEP, and GP

Polyphenol analysis revealed that the weight percentages of polyphenols in the EP and NEP fractions were 2.26% and 6.91% respectively. The EP fraction contained 180.0 mg/g of total phenolics and the NEP fraction contained 10.5 mg/g of total phenolics. Total anthocyanin and proanthocyanidin content of the EP fraction was 37.8 mg/g and

305.5 mg/g, respectively, whereas these polyphenols were not detectable in the NEP fraction (**Table 4.2**). Individual anthocyanin analysis revealed that the most abundant anthocyanins present in the EP fraction were peonidin-3-O-glucoside (36.6 u/mg), malvidin-3-O-glucoside (30.8 u/mg), and peondin-3-O-cis (6"-O-p-coumaryl)-glucoside (28.5 u/mg) (**Table 4.3**). The profile of the degree of polymerization of the proanthocyanidins in the EP fraction is shown in (**Fig. 4.1**).

EP fraction with or without NEP fraction lowers body fat gain

Body weight gains and energy intakes were greater in all HF-fed mice compared to the LF control mice (**Table 4.4**). There was no difference in the energy intake between all of the HF-fed mice, with the exception of the HF-EP+NEP group that consumed approximately 10% fewer calories than the HF controls (Table 4.4). Mice fed the HF-EP and HF-EP+NEP diets had lower body weight gains (Table 4.4; ~28% and 40%, respectively) and body fat percentages than the HF controls (**Fig. 4.2;** 27% and 37%, respectively). Mice fed the HF-EP, HF-NEP and HF-EP+NEP diets had lower total WAT depot weights compared to HF controls (Fig.4.2). Paradoxically, mice fed the HF-GP diet did not have improvements in body fat percentage, body weight, and total WAT depot weights compared to the HF controls.

EP fraction improves glucose disposal, insulin resistance, and hypertriglyceridemia

To assess the impact of grape consumption on glucose disposal and insulin resistance, GTTs were conducted at weeks 7, 12, and 16, and fasting plasma insulin and HOMA-IR

were measured at week 16, respectively. Mice consuming the HF control diet had impaired GTTs at all three time points compared to the LF controls (**Fig. 4.3A**). This HF diet impairment was attenuated at all three time points in mice fed the HF-EP diet. Mice fed the HF-NEP and HF-EP+NEP diets had improved GTTs at weeks 12 and 16 compared to the HF controls. HOMA-IR index calculations at week 16 indicated that mice consuming the HF-EP+NEP fraction had improved insulin sensitivity compared to the HF controls (**Fig. 4.3B**). Surprisingly, consuming grapes did not significantly improve GTT, fasting plasma insulin, and HOMA-IR measurements. Mice fed the HF-EP and the HF-EP+NEP diets had lower plasma TG levels compared to the HF-fed controls (**Fig. 4.3C**).

Grape fraction consumption lowers hepatic TG levels and alters the expression of genes associated with lipid metabolism and inflammation

Liver tissue was analyzed to assess the impact of the diets on liver TG levels and markers of lipogenesis, lipolysis, and fatty acid oxidation. Mice consuming the HF control diet had greater liver weights, TG content, and mRNA levels of the lipogenic genes *Pparγ* and *Fas* compared to the LF-fed mice (**Fig. 4.4**). Compared to LF controls, HF feeding also increased gene expression of stearoyl regulatory element binding protein 1 (*Srebp-1c*), and stearoyl-CoA desaturase 1 (*Scd1*), which was not effected by whole grape or grape fraction feeding (Figure 4.4). Mice consuming the HF-NEP and HF-EP+NEP diets had lower liver weights and mice consuming the HF-EP+NEP diet had lower liver TG content compared to HF controls. Mice fed the HF-EP and HF-EP+NEP

diets had greater mRNA levels of hormone sensitive lipase (*Hsl*), while mice fed the HF-EP+NEP diet had increased mRNA levels of adipose triglyceride lipase (*Atgl*) compared to HF controls (Figure 4.4). HF feeding increased the hepatic gene expression of the inflammatory marker C-reactive protein 1 (*Crp1*), which was attenuated by HF-EP and HF-EP+NEP feeding (Figure 4.4). Interestingly, all mice consuming HF diets had ~60% lower mRNA levels of phosphoenolpyruvate carboxykinase (*Pck1*), a marker of gluconeogenesis. No treatment differences in the expression of carnitine palmitoyltransferase 1-a (*Cpt1a*) were detected (data not shown). Hepatic oil-red-o staining indicated greater lipid content in the livers of mice fed the HF control diet compared to the LF controls, which was attenuated by HF-EP, HF-NEP, and HF-EP+NEP consumption (**Fig. 4.5**).

EP fraction with or without NEP fraction improves the expression of WAT genes associated with inflammation and lipid metabolism

To determine if the negative metabolic effects caused by HF-feeding were associated with inflammation in WAT, mRNA levels of several proinflammatory genes were measured in inguinal (subcutaneous) and epididymal (visceral) WAT. In inguinal WAT, mice fed the HF control diet had greater mRNA levels of cluster of differentiation 11c (*Cd11c*) and monocyte chemoattractant protein 1 (*Mcp1*) compared to LF controls (**Fig.4.6A**). This HF-induced increase was attenuated in mice fed the HF-EP and HF-EP+NEP diets. In epididymal WAT, HF-feeding increased mRNA levels of *Cd11c*, *Mcp1*, tumor necrosis factor alpha (*TNFα*), and epidermal growth factor-like module

containing mucin-like hormone receptor 1 (*Erm1; F4/80* human orthologue) compared to LF controls (**Fig. 4.6B**). Consistent with the inguinal data, mice fed the HF-EP and HF-EP+NEP diets had lower mRNA levels of these genes compared to the HF controls.

To determine whether the reduction in adiposity observed with feeding of HF-EP, HF-NEP, and HF-EP+NEP diets was associated in alterations in lipid metabolism, inguinal and epididymal WAT were examined for markers of; (i) lipogenesis (i.e., *Pparγ*, *Srebp1c*, *Scd1*, acylglycerol-3-phosphate-O-acyltransferase 2 (*Agpat*2), fatty acid synthase (*Fas*), glycerol-3-phosphate acyltransferase2 (*Gpat2*)), (ii) lipolysis (i.e., *Atgl* (iii) beta-oxidation (i.e., carnitine palmitoyltransferase 1-b (*Cpt1b)*, *Pparα*), (iv) SCFA receptors (i.e. *GPR43*), and (v) plasminogen activator inhibitor-1 (*Pai-1*). In inguinal WAT, mice fed the HF diet had greater expression levels of *Agpat2,* which were lower in the HF-EP and HF-EP+NEP groups (**Fig. 4.7A**). *Cpt1b* gene expression was lower in mice fed the HF-EP, HF-EP+NEP diets compared to the HF-fed mice. No treatment differences in gene expression were observed for *Srebp1c*, *Scd1*, *Fas*, *Gpat2*, *Atgl*, and *Pparα.*

In epididymal WAT, mice consuming the HF diet had higher mRNA levels of *Agpat2,* a marker of TG biosynthesis, compared to LF controls (**Fig. 4.7B**). Feeding HF-EP and HF-EP+NEP attenuated this HF-induced increase in *Agpat2* expression. Additionally, mice fed the HF diet-had lower levels of *Pparγ* compared to LF controls. The expression of *Pparα,* a marker of beta oxidation, was reduced in mice fed the HF control and the GP diets compared to LF controls. Surprisingly, HF feeding increase the mRNA levels of *Cpt1b,* beta-oxidation marker, which was reduced by HF-EP and HF-EP+NEP feeding. Additionally, mice fed the HF-EP+NEP diet had lower mRNA levels of *GPR43,* a SCFA-

activated receptor associated with increased energy harvest, compared to HF- and LF-fed controls. No differences in gene expression in epididymal WAT were observed for *Srebp-1c*, *Scd1*, *Fas*, *Gpat2*, and *Atgl* (data not shown).

Minimal influence of HF-feeding and grape fraction consumption on markers of intestinal inflammation

Given the reported adverse effects of consuming saturated fats [reviewed in 10] on intestinal health, and potential prebiotic impact of grapes, we measured the effects of the diets on markers of intestinal inflammation and barrier function. For inflammatory status, the mRNA levels of *Cd11c*, *Cd68*, *Erm1*, *Mcp1*, *Tnfα*, *Tlr4*, and interleukin (*Il*)*1b* in ileum and proximal colon mucosa and the activity of ileal myeloperoxidase were measured. Unexpectedly, the mRNA levels of proinflammatory genes were not increased in the ileal mucosa of HF- fed mice compared to the LF controls. Equally surprising, mice consuming the HF-GP diets had greater mRNA levels of *Tlr4* and *Cd68* compared to the HF-fed mice (**Fig. 4.8A**). Although the activity of myeloperoxidase, an enzyme expressed neutrophils and indicative of neutrophil infiltration, was ~50% lower in the ileal mucosa of mice consuming the HF-EP and HF-EF+NEP diets compared to the HF-fed mice, these differences were not statistically significant (**Fig. 4.8A**). Similarly, the expression of *Tlr4* was greater in the colonic mucosa of HF- GP fed mice compared to the HF controls (**Fig. 4.8B**). Two of the proinflammatory genes measured in the colonic mucosa where increased by HF feeding (i.e., *Cd11c*, *Cd68*) and only *Cd68* was attenuated in mice consuming the HF-EP+NEP diet (Fig. 4.8B).

Influence of grape fractions on intestinal L-cell gene expression

Intestinal microbial metabolites impact local and systemic health, in part, by influencing fermentation products (e.g., SCFA), energy harvest, and the release of endocrine signals from L-cells located in the mucosa epithelium. Locally, these metabolites influence the integrity of the mucosa barrier and systemically impact energy balance and carbohydrate and lipid metabolism [20]. Therefore, we determined the influence of the dietary treatments on the induction of genes associated with SCFA receptors (e.g., *Gpr41, -43, and -119*) and two of their downstream targets (e.g., *Glp* and *Pyy*) in the ileum and proximal colon. Mice fed the HF-EP, HF-NEP, and HF-EP+NEP diets had greater mRNA levels *Gpr43,* but not *Gpr41,* in the ileum mucosa compared to the HF controls (**Fig. 4.9A**). Mice fed the HF-EP+NEP and the HF-GP diets had lower mRNA levels of *Gpr41* in the ileum mucosa compared to the HF controls. Mice fed the HF-GP diet has the highest levels of *Glp* compared to all other treatments (Fig.4.9A). In contrast, mice fed the HF diet had higher mRNA levels of *Gpr43* in the proximal colon compared to the HF-NEP, HF-EP+NEP, and HF-GP groups (**Fig. 4.9B**). Lastly, HF-feeding increased *Pyy* mRNA levels in the proximal colon, which were lower in mice fed the HF-EP+NEP diet.

Discussion

Rationale and significance of this study

We previously demonstrated that feeding whole GP in conjunction with a HF diet (i.e., 34% kcals from fat; 3% from soybean oil and 31% from milk fat, which was similar to the amount of fat consumed by Americans in the 50th percentile [33] for 10 weeks

modestly reduced adiposity (3% and 5% GP), hepatic steatosis (5% GP), and the ileal mucosa abundance of the deleterious sulfidogenic bacterium species *DSB* and a sulfidogenic gene *drsA-Bw* (3% GP), and increased the proximal colon mucosa abundance of the beneficial bacterium *Akkermansia muciniphila* (5% GP) and the localization of the tight junction protein ZO-1 on the apical epithelial surface of the ileum (3% and 5% GP). However, consuming the HF diet for 10 weeks only modestly increased adiposity, insulin resistance, and several markers of inflammation in epididymal (visceral) WAT, and had no effects on markers of intestinal inflammation. Therefore, we conducted the current 16 week study using a higher level of dietary fat (i.e., 45% kcals) and a fatty acid profile that was similar to Americans in the 75th percentile (i.e., soybean oil, butter, lard, shortening, and beef tallow [33] to enhance the metabolic consequences of diet-induced obesity. We also wanted to know which fraction of GP was responsible for reducing adiposity and its potential metabolic complications. Therefore, we examined the effects of whole GP (i.e., 5%) and two isolated fractions of grapes (i.e. methanol derived EP and NEP fractions which were fed at amounts equal to those in 5% GP) on the development of obesity, steatosis, glucose intolerance, and WAT inflammation in C57BL/6J mice fed a HF diet similar to Americans. The EP fraction contained ~ 16 times more polyphenols, particularly anthocyanins (i.e., glucosides of anthocyanidins; Table 4.3) and proanthocyanidins (i.e., polymers of anthocyanidins formed by the condensation of flavans; Table 4.2) , than the NEP fraction. The results indicate that in the context of HF feeding, consumption of the polyphenol-rich, EP fraction, alone or in combination with the NEP fraction, attenuated diet-induced obesity,

insulin resistance, steatosis, and chronic inflammation in WAT. However, consuming whole GP had no beneficial impact on the outcomes measured, and reasons for this lack of an effect are unclear.

Potential mechanisms by which anthocyanin-rich EP fraction suppresses adiposity and inflammation and improves insulin sensitivity

Anthocyanins and proanthocyanidins are groups of flavonoids present in relatively large amounts in table grapes. Anthocyanins give these berries their unique colors. Our phenolic analysis indicated that the most abundant anthocyanins found in California table grapes are malvidin-3-O-glucoside, peonidin-3-O-glucoside, and cyanidin-3-O-glucoside. While studies have demonstrated anti-obesity and anti-diabetic effects of anthocyanins [reviewed in 41], studies focusing on the effects of feeding whole grapes and grape fractions feeding in subjects consuming a diet similar in fat amount and types to the American diet are lacking. Consistent with our anthocyanin-rich EP data, mice consuming a HF diet (i.e., 60% kcals from fat, primarily lard) supplemented with anthocyanin-rich muscadine grape or wine extracts for 15 weeks had lower body weights, plasma glucose and TG levels, insulin resistance, and CRP levels compared to controls [42]. Also, dietary supplementation of grape seed procyanidins has been demonstrated to prevent body weight gain, reduces WAT inflammatory markers (i.e. *Tnfα*, *Il-6*, *Crp*) in high-fat-fed rats [43]. Similar effects were observed with grape seed extract supplementation including reductions in weight gain, WAT weights and blood lipid levels in HF fed mice [44], and improvements insulin sensitivity and reductions in oxidative stress in high-fructose fed rats [45]. These findings are consistent with our data

demonstrating that EP-fed mice had less body weight gain, reduced WAT weights, lower plasma TG, improved insulin sensitivity, and reduced markers of inflammation in WAT (i.e. *Cd11c*, *Mcp1*, *F4/80*).

While research is lacking on the effects of grape anthocyanins specifically, anthocyanins from other dietary sources have been well documented in the literature. Anthocyanins have been demonstrated to consistently exert anti-inflammatory and anti-obesity effects in vitro, and in some instances in vivo. For example, anthocyanins from black soybeans reversed weight gain, reduced the levels of serum TG and cholesterol, and increased the levels of high-density lipoprotein (HDL) [46]. These findings are consistent with our data demonstrating that mice fed the anthocyanin-rich EP fraction had reduced body weight gain and plasma TG levels compared to the HF controls. Also, anthocyanins from purple corn prevented HF diet induced increases in body weight and adipose tissue weights in mice [47]. Similarly, in HF (60% kcals from lard) fed mice, purified anthocyanins from blueberries prevented body weight gain and fat gain compared to controls [48]. Additionally, tart cherries, similar to grapes with respect to anthocyanin profile, reduced fat mass and WAT markers of inflammation including Il-6 and Tnfα. [49]. These data are consistent with our data demonstrating that the anthocyanin-rich EP fraction decreased body weight gain, fat mass, plasma TG levels, and WAT expression of inflammatory markers including *Tnfα*, *Mcp1*, *Cd11c*, *F4/80*. Additionally, 3T3-L1 preadipocytes treated with anthocyanins had decreased TG accumulation and gene and protein expression of *Pparγ* and *Fas* [50]. While we did not

observe alterations in expression of *Pparγ* and *Fas* within the WAT, we did observe that mice fed the EP fraction had reduced expression of the lipogenic gene *Agpat2*.

We also demonstrated in this study that mice fed the anthocyanin-rich EP fraction had improved glucose tolerance and insulin sensitivity compared to the HF controls. Similar effects of improvements in glucose tolerance were observed with supplementation of anthocyanins from Maqui Berry in HF fed mice [51] and also with supplementation of anthocyanins from Cornelian cherry in HF fed mice [52]. It has been reported that cyanidin-3-O-glucoside supplementation improves insulin sensitivity in diabetic mice via down-regulation of retinol binding protein and up-regulation of GLUT4 gene expression [53]. This could be a potential mechanism by which the EP fraction improved glucose tolerance and insulin sensitivity.

One novel finding of our study was that the anthocyanin-rich EP fraction increased expression of *Hsl*, a gene associated with lipolysis, and reduced expression the lipogenic-related gene, *Pparγ* within the liver. This indicates that the EP fraction may reduce steatosis through up-regulation of lipolytic pathways and suppression of lipogenic pathways. Further research examining the effect of the EP fraction on these pathways would be beneficial to better understand the mechanisms by which the EP fraction reduces adiposity or steatosis.

Relationship of intestinal inflammation to systemic inflammation

High fat feeding is known to increase body fat mass and has been shown to consistently induce chronic, low grade inflammation systemically [reviewed in 54].

However, the link between diet and intestinal inflammation has not been as extensively studied. In the current study, HF feeding did not increase markers of inflammation in the ileum or proximal colon after 16 weeks, nor was there any beneficial effect of GP or grape fraction consumption. However, other research has shown that HF diets can trigger intestinal inflammation or endotoxemia that may increase systemic inflammation, possibly due to intestinal dysbiosis and barrier dysfunction [7-13]. For example, mice fed a HF diet had increased levels of saturated fatty acids and lipopolysaccharide (LPS) within intestinal tissue samples resulting in increased *Tnf-α* and *Il-6* expression through activation of the toll-like receptor (Tlr)4/(nuclear factor)Nf-κB signaling pathway [18]. Mice fed a HF diet were also more susceptible to dextran sulfate sodium (DSS) induced colitis [19]. As a result the immune response was reduced which was associated with an increase in non-CD1d-restricted natural killer T (NK T) cells, which produce inflammatory cytokines similar to those secreted by adipocytes, decreasing the number of regular NK T cells normally found in the colon. In addition, dietary supplementation with bioactive food components such as grape seed extract and red wine extracts has been shown to improve the intestinal health of subjects fed HF diets. For example, chronic consumption of grape seed extract increases tight junction protein and reduces fecal calprotectin; a neutrophil protein used as a marker of intestinal inflammation [55], and attenuates inflammatory signaling in chemically-induced colitis [56]. Furthermore, in Wistar rats treated with concentrated grape juice, induced colitis was attenuated [57]. In the current study, consumption of the EP and EP+NEP fractions notably attenuated several proinflammatory markers in the WAT, but only EP+NEP decreased the

expression of the macrophage markers *Cd68* in the proximal colon. Although the activity of the neutrophil enzyme myeloperoxidase was decreased by 40-50% in the EP and EP+NEP groups compared to the HF controls, these differences were not statistically significant. However, measuring the protein levels of inflammatory markers and the activity of myeloperoxidase and alkaline phosphates in the colon may provide more insight. Alternatively, the intestinal linkage to systemic effects of HF and grape fraction feeding may be due to changes in gut microbiota, as indicated in our first study (Chapter III).

The intestinal microbiota is highly influenced by environmental changes which have been linked to alterations in host health and energy intake. Research has shown that alteration to the gut microbiota by HF feeding can result in increased adiposity, insulin resistance and increased inflammatory cytokines and chemokines [58 -60]. This has been linked to impaired gut barrier function (i.e., the inhibition of tight junction protein production or reduction of mucin secretion from goblet cells) leading to endotoxemia [61,62] and impaired regulation of energy intake and metabolism through activation TLR-dependent enteroendocrine signaling pathways resulting in impaired neural response to leptin [reviewed in 63]. Conversely, microbial consumption of dietary fiber and polyphenols such as those found in grapes, may reduce intestinal and systemic inflammation through regulation of energy intake and improved intestinal barrier function [reviewed in 64]. Grape polyphenols in particular may have a major impact on the microflora of the large intestine. For example, red wine grape polyphenols given to humans for 4 weeks significantly increased the number of *Enterococcus, Prevotella,*

Bacteriodetes, and *Bifidobacterium* bacteria which correlated to improved blood pressure, and serum levels of TG, total cholesterol, and CRP [65]. Further evidence of the prebiotic effects of grape consumption can be seen in rats given grape pomace juice which increased fecal counts of *Lactobacillus* and *Bifidobacterium* which consequently also resulted in an increase in the concentration of primary bile acids, cholesterol, and cholesterol metabolites while decreasing the concentration of secondary bile acids [66]. This suggests that alteration of the microflora by grape polyphenols may inhibit cholesterol absorption thus improving circulating levels. Indeed, fermentation of fiber and polyphenols by colonic microbes into SCFA has been shown to regulate energy intake and metabolism through stimulating the release of glycoproteins (e.g., Glp-1, Glp-2, and Pyy) by activating Gpr43 and Gpr41 on enteroendocrine cells [reviewed in 67]. Butyrate in particular is also beneficial to intestinal homeostasis as an endothelial energy source as well as through increasing tight junction protein synthesis and mucin secretions [68,69]. In the current study, the expression of several markers of enteroendocrine cell secretions associated with SCFA activation were upregulated. However, these results were conflicting between the ileum (i.e., *Gpr43* mRNA levels increased by the EP, NEP, EP+NEP diets) and the proximal colon (i.e., *Gpr43* mRNA levels increased by HF diet only). Future analysis of the intestinal microbiota composition, protein levels of Gprs, Pyy, and Glp1/2 levels, and gut barrier function should provide more insight about the extent to consuming HP, GP, and grape fractions influences these intestinal biomarkers.

Limitations and unanswered questions

The beneficial systemic effects of consuming the EP grape fraction may be due to its rich anthocyanin content. However, the whole GP diet did not have the same positive outcomes as the EP or NEP diets. There were several differences in the design of our previous (Chapter III) and current study that may have contributed to these conflicting results. The previous research was shorter (10 weeks) compared to the current study (16 weeks) and the diet composition with regards to fat (i.e., primarily butter fat at 34% kcals versus a combination of butter, lard, shortening, and beef tallow at 45% kcals from fat, respectively) and fiber (50 g versus 40 g cellulose, respectively). The additional 6 weeks in length of the current study may have caused any potential health benefits of the whole grape diet to have diminished. Yet, by week 10 of the second study, there were no differences between the HF and GP group in regards to body weight, body fat percentage, and glucose tolerance. Furthermore, there may have been a different interaction between the GP and the four types of fats in the current study versus the sole fat source (butter) in the first study (i.e., the polyphenols in the whole GP may interact with butter differently than with the other fat sources, thereby affecting their bioaccessibility and bioavailability). Indeed, the fat content of the diet can differentially impact polyphenol digestion and absorption [70]. Such an interaction may have enabled the polyphenols to inhibit lipase activity, possibly affecting the rate of fat absorption. The increase in calories in the current study may have been too excessive for the beneficial effects of the whole GP to have a positive impact on lipid absorption as well. In order to increase the percentage of fat in the current study, the amount of cellulose and corn starch were

reduced compared to the previous study. The alteration in carbohydrate and fiber content may have resulted more time for increased absorption of macronutrients. Such a scenario would result in greater energy harvest, adipogenesis, and triglyceride synthesis in the liver and WAT, independent of any effects by the grape powder. Furthermore, the extraction process of the anthocyanin rich fraction may have increased the bioaccessibility and subsequent bioavailability of the polyphenols within the powder by liberating them from interactions within the food matrix, making them more susceptible to interactions with brush boarder enzymes potential enhancing their absorption in the small intestine. Similarly, liberation of the polyphenols may have altered the luminal environment, thereby improving the confluence of beneficial bacteria.

Another possible impact of the reduced fiber may have been on the microbiota of the large intestine, as fiber is a primary energy source for a vast majority of microbes in this environment. As such, the reduced fiber may impact the production of SCFAs as well as reduce the diversity of the microbiota, which typically results in intestinal inflammation, impaired barrier function, or adverse systemic effects. Interestingly, although the GP diet had no positive health impact in the second study, the extractable polyphenol fraction did have a positive effect on reducing HF-mediated adiposity and systemic inflammation. Future studies should focus on feeding single anthocyanins and there combinations (i.e., candidates in Table 4.3) in conjunction to a HF diet to see which may be the most effective.

Acknowledgements

We would like to acknowledge the contributions of everyone involved in collecting data for this study. Brian Collins and Jessie Baldwin equally conducted all stages of the animal study, including caring, feeding, and weighing of the mice, the glucose tolerance testing, and the measurement of body fat using DEXA. Brian Collins additionally measured markers of ileal and colonic gene and protein expression associated with inflammation, ileal activity of myeloperoxidase. Jessie Baldwin additionally measured markers of inguinal and epididymal gene and protein expression associated with inflammation, fat depot and liver gene expression of markers of lipolysis and lipogenesis, and serum triglyceride levels. Robin Hopkins measured plasma insulin using an ELISA assay. Mary-Ann Lila and Mary Grace together performed the methylated extraction process to produce the EP and NEP fraction and measured the concentration and composition of the polyphenols present. Chia-Chi Chuang measured liver triglyceride levels and Oil-Red-O liver staining. Paula Cooney measured the body fat mass using DEXA (with the assistance of Brian Collins and Jessie Baldwin).

Table 4.1. Diet Formulations

Ingredients (gram)	LF	HF	GP	EP	NEP	EP+NEP
Casein	200	200	200	200	200	200
L-Cystine	3	3	3	3	3	3
Corn Starch	306.2	73.2	73.2	73.2	73.2	73.2
Maltodextrin 10	125	94	94	94	94	94
Fructose	0	21.9	0	21.9	21.9	21.9
Dextrose, Monohydrate	0	21.9	0	21.9	21.9	21.9
Sucrose	68.8	139	139	139	139	139
Cellulose	50	40	40	40	40	40
Soybean Oil	25	48.8	48.8	48.8	48.8	48.8
Butter	0	44.7	44.7	44.7	44.7	44.7
Lard	20	35.3	35.3	35.3	35.3	35.3
Shortening	0	55.6	55.6	55.6	55.6	55.6
Beef Tallow	0	20.3	20.3	20.3	20.3	20.3
Mineral Mix	10	10	10	10	10	10
DiCalcium Phosphate	13	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5	5.5
Potassium Citrate	16.5	16.5	16.5	16.5	16.5	16.5
Vitamin Mix	10	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2	2
NEP Grape Fraction	0	0	0	0	2.97	2.97
EP Grape Fraction	0	0	0	0.97	0	0.97
Grape Powder	0	0	43	0	0	0
Total	1055.1	854.7	857.5	855.7	857.7	858.7

*LF, low fat control; HF, high fat sugar control; GP, grape powder; EP, extractable polyphenol fraction; NEP, non-extractable polyphenol fraction

Table 4.2. Phenolic Composition of Grape Powder (GP), Extractable Polyphenol (EP), and Non-Extractable Polyphenol (NEP) Fraction

Assay	**GP**	**EP**	**NEP**
Weight percentage (%)	100	2.26	6.91
Total phenolics[1]	9.13	180.0 ± 1.3	10.5 ± 1.02
Total anthocyanins[2]	0.33	37.8 ± 1.8	ND
Total proanthocyanidins[3] (mg/g)	3.05	305.5 ± 7.0	ND

[1]Total phenolics, mg/g DM as gallic acid equivalent (Folin Ciocaleau assay)

[2]Total anthocyanins, mg/g DM as cyaniding glucoside equivalent (pH differential assay)

[3]Total proanthocyanidins, mg/g DM as cyaniding glucoside equivalent (DMAC assay)

Table 4.3. Anthocyanin Concentration in Grape Powder (GP) and the Extractable Polyphenol (EP) Fraction.

Peak #	**Identification**	**GP Concentrations** (mg/kg)	**EP Concentrations** (µg/mg)
1	Delphinidin-3-*O*-glucoside		4.93
2	Pyanidin-3-*O*-glucoside		10.56
3	Petunidin-3-O-glucoside		4.91
4	Peonidin-3-O-glucoside		36.56
5	Malvidin-3-O-glucoside	145.2	30.83
6	Malvidin-3-O-(6"-O-acetyl-glucoside)		3.70
7	Petunidin-3-O-cis-(6"-p-coumaryl-glucoside)		2.56
8	Cyanidin-3-O-(6"-O-p-coumaryl-glucoside)	125.0	2.21
9	Petunidin-3-O-trans-(6"-O-p-coumaryl-glucoside)		3.24
10	Peonidin-3-O-cis-(6"-p-coumaryl-glucoside	31.7	2.73
11	Malvidin-3-O-cis(6"-p-coumaryl-glucoside		2.83
12	Peonidin-3-O-cis-(6"-O-p-coumaryl-glucoside)		28.460
13	Malvidin-3-O-trans-(6"-O-p-coumaryl-glucoside)		4.93
	Total anthocyanins		133.52

Table 4.4. Body Weight Gain (BWG), Food Conversion Efficiency (FCE), and Total Calorie Intake per Treatment Group.*

Diets	**BWG** (g)	**FCE** (per cage, g food/g BW)	**Kcal Intake** (per cage)
LF	11.4 ± 0.5^{d}	28.74 ± 1.24^{a}	2502.26 ± 82.01^{c}
HF	24.8 ± 1.0^{a}	13.71 ± 0.35^{cd}	3253.91 ± 99.43^{a}
EP	17.8 ± 0.9^{b}	18.43 ± 0.46^{b}	3135.50 ± 165.40^{ab}
NEP	20.2 ± 0.9^{b}	15.98 ± 0.63^{c}	3064.54 ± 121.38^{ab}
EP+NEP	15.0 ± 0.9^{c}	20.71 ± 1.21^{b}	2914.92 ± 63.30^{b}
GP	24.9 ± 1.0^{a}	13.26 ± 0.31^{d}	3144.70 ± 97.74^{a}

*LF, low fat control; HF, high fat sugar control; GP, grape powder; EP, extractable polyphenol fraction; NEP, non-extractable polyphenol fraction.

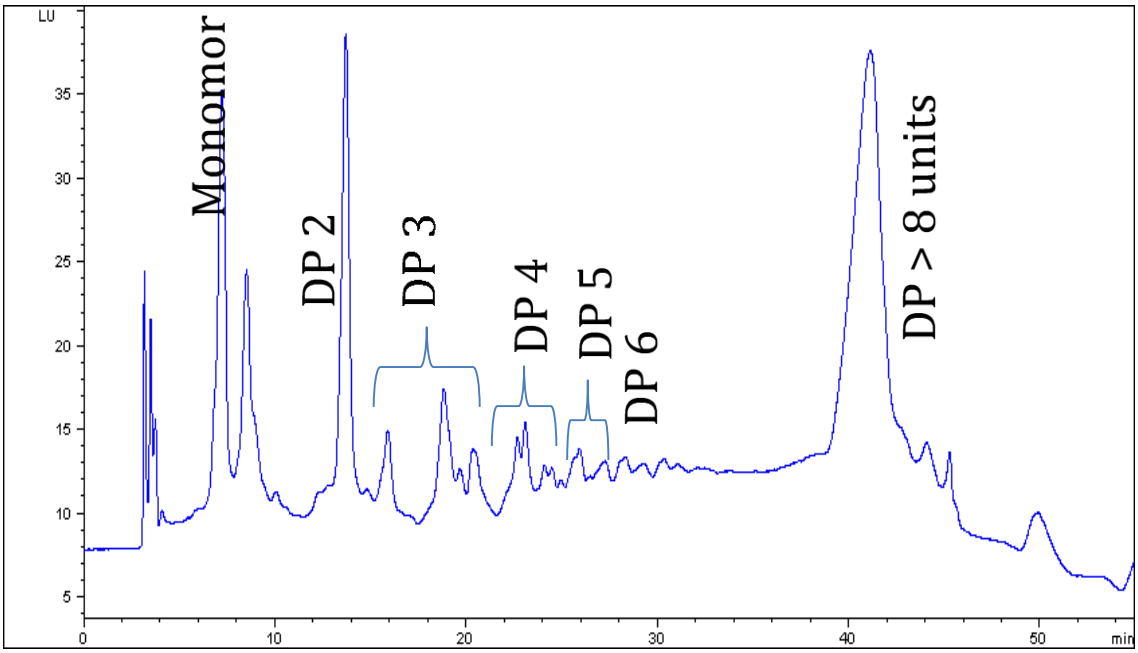

Figure 4.1. Proanthocyanin Degree of Polymerization in EP Fraction. NP-HPLC-FLD chromatogram (excitation 230 nm, emission 320 nm) for proanthocyanidins showing degree of polymerization (DP) in extractable polyphenol (EP) from grapes.

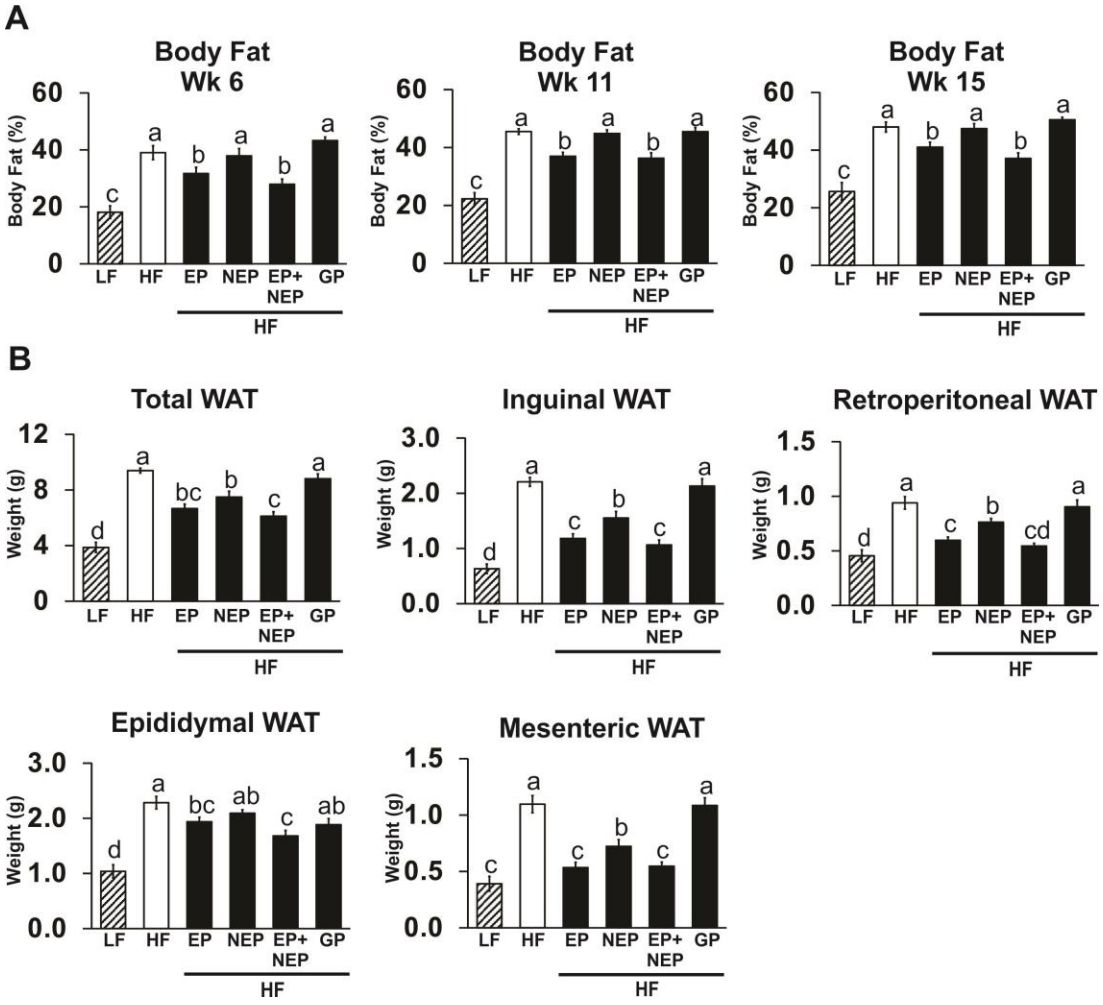

Figure 4.2. Body Fat Percentages and WAT Depot Weights. Adiposity indices of C57BL/6J mice fed a low fat (LF),high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. Body fat percentages were measured at weeks 6, 11, and 15 using dual energy x-ray absorptiometry (DEXA). At week 16, the inguinal, retroperitoneal, epididymal, and mesenteric white adipose tissue (WAT) depots were excised and weighed. The weights of the inguinal, retroperitoneal, epididymal, and mesenteric depots were measured, and their sum labelled total WAT. Means ± SEM (n = 9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's test.

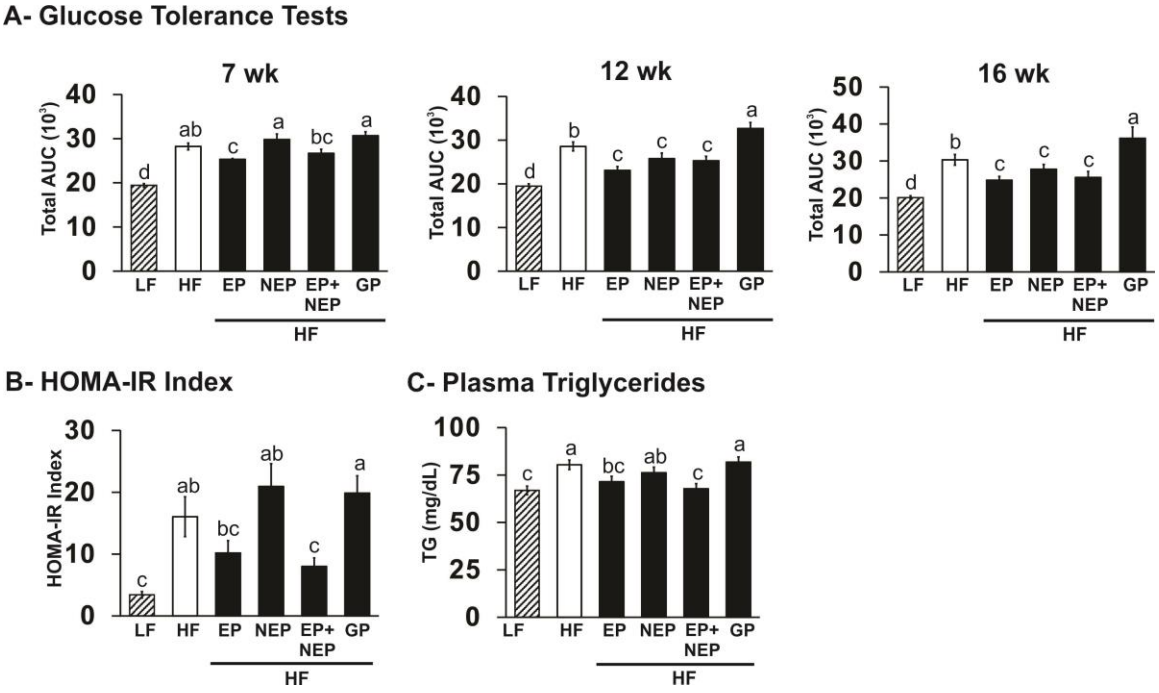

Figure 4.3. Glucose Tolerance Tests, HOMA-IR Index, and Plasma Triglycerides
(A)Glucose tolerance tests (GTT)s , (B) the homeostatic model assessment of insulin resistance (HOMA-IR), and (C)plasma TG levels of C57BL/6J mice fed a low fat (LF),high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. At weeks 7, 12, and 16, GTTs were conducted on mice fasted for 8 h and injected i.p. with a 20% glucose solution. Fasting plasma insulin was collected at week 16 and used to calculate the HOMA-IR index. Data are expressed as total area under the curve (AUC) for the GTTs. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's test.

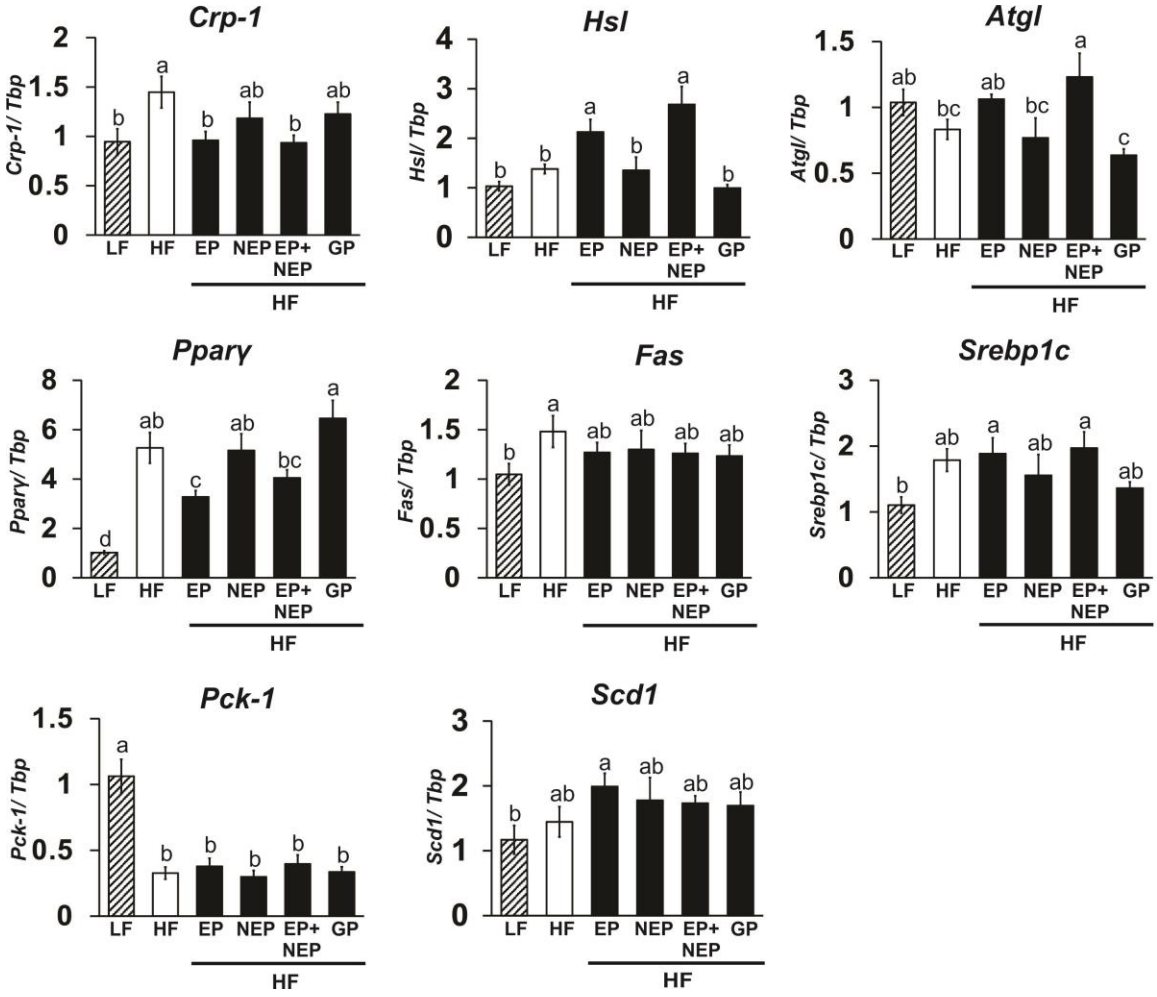

Figure 4.4. Liver Markers of Inflammation and Lipid Metabolism. Liver gene expression of markers of inflammation, lipolysis, and lipogenesis in liver of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. qPCR was conducted to measure mRNA abundance of genes associated with hepatic inflammation, lipolysis, or lipogenesis. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's test.

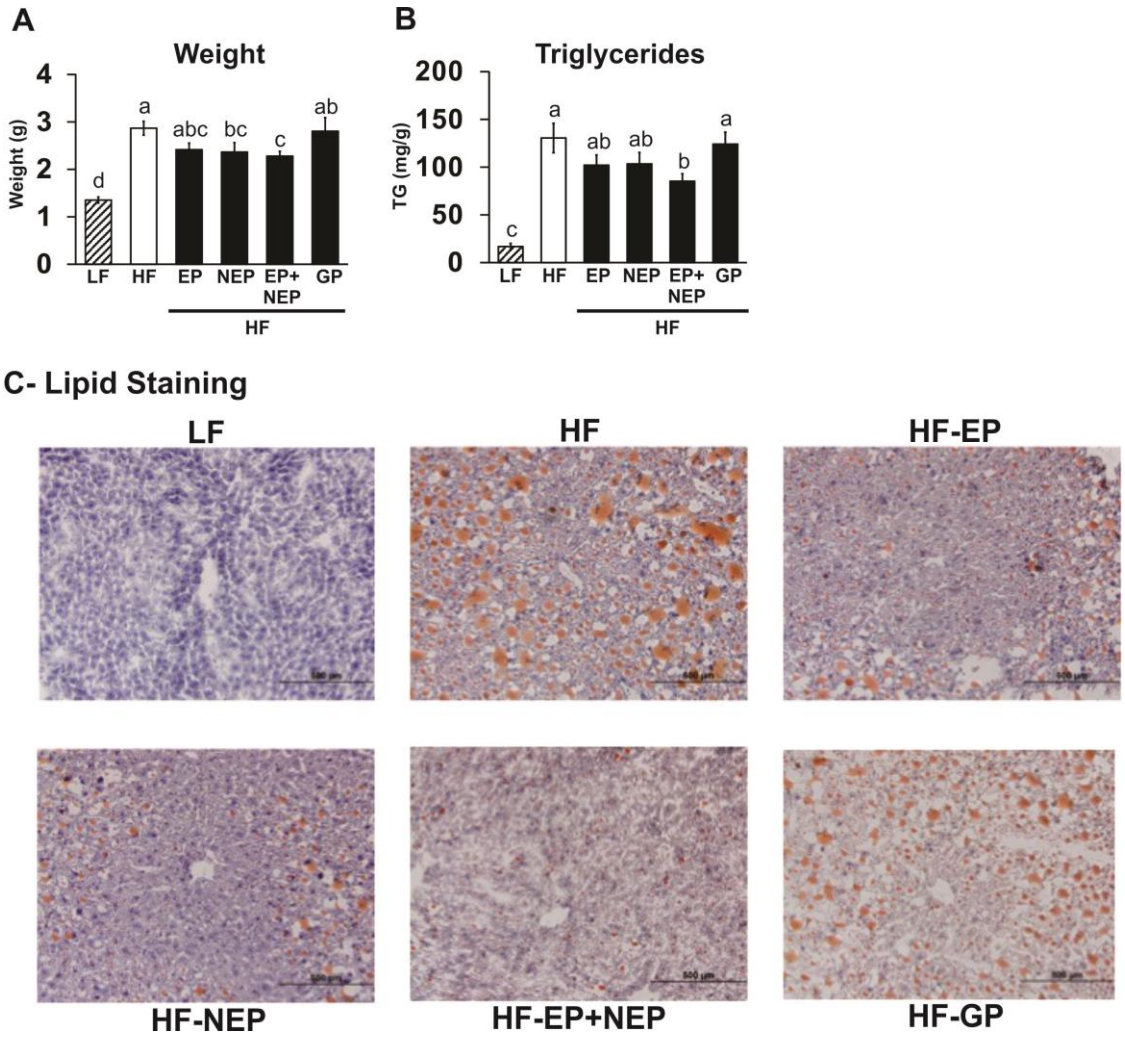

Figure 4.5. Liver Weight, Triglycerides, and Oil Red O Staining. (A) Liver weights, (B) liver triglyceride content, and (C) Oil red O staining of liver tissue of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. At week 16, liver tissues were excised and frozen in OCT compounds, cut at 5 μm, mounted on slides, and stained with Oil red O solution. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's test.

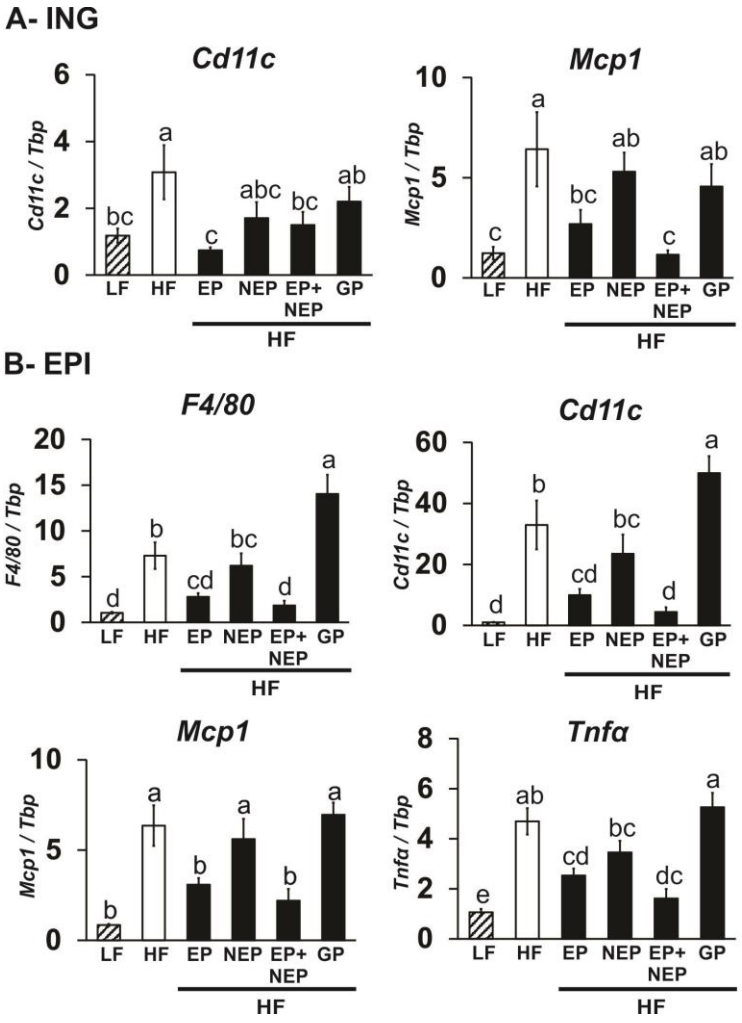

Figure 4.6. Expression of Markers of Inflammation in WAT. The expression of markers of inflammation in (A) inguinal and (B) epididymal WAT of C57BL/6J mice fed a low fat (LF),high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for qPCR was conducted to measure mRNA abundance of genes associated with inflammation in inguinal (A; subcutaneous) and epididymal (B; visceral) WAT depots. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's test.

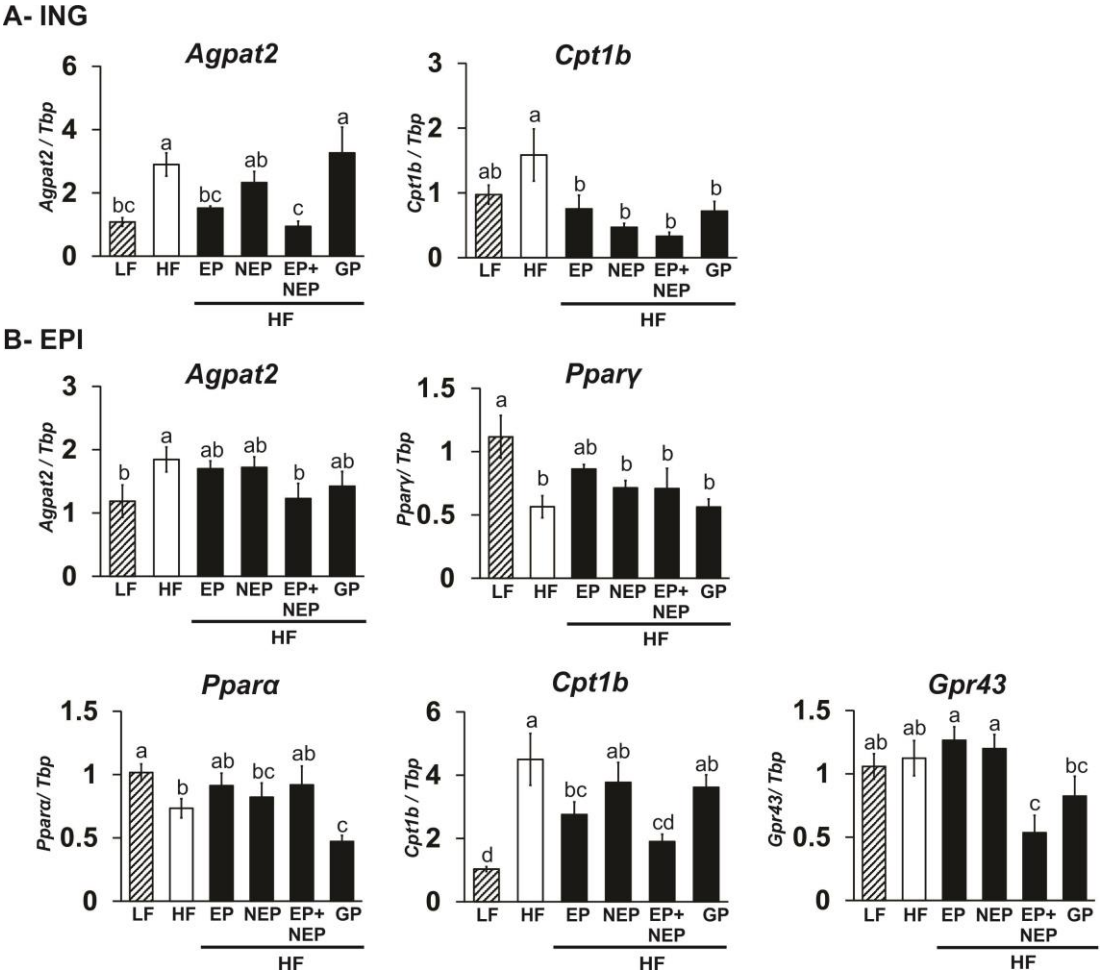

Figure 4.7. Expression of Markers of Lipid Metabolism in WAT. The expression of markers of lipogenesis, lipolysis, or fatty-acid oxidation in (A) inguinal and (B) epididymal WAT of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. qPCR was conducted to measure mRNA abundance of genes associated with lipogenesis, lipolysis, and fatty-acid oxidation in inguinal (A; subcutaneous) and epididymal (B; visceral) WAT depots. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's test.

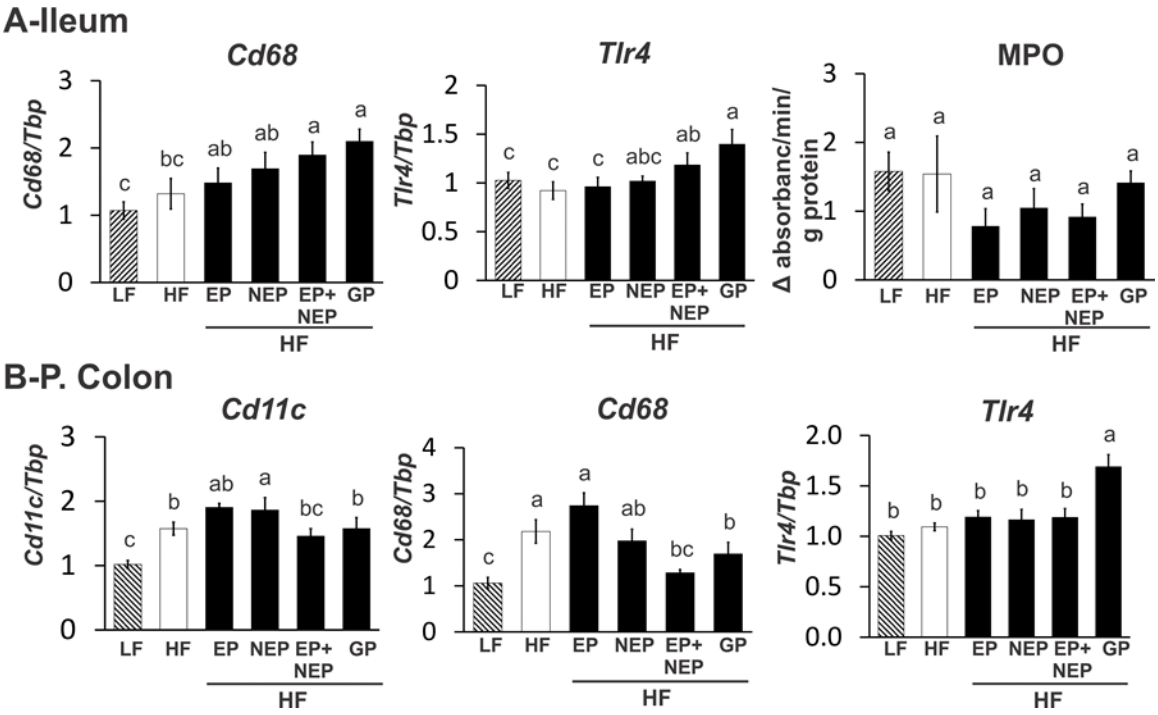

Figure 4.8. Expression of Markers of Intestinal Inflammation. The expression of markers of inflammation in the (A) ileum, (B) proximal colon, and the (A) activity of myeloperoxidase in the mucosa of C57BL/6J mice fed a low fat (LF),high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. qPCR was conducted to measure mRNA abundance of genes associated with inflammation in ileum mucosa and proximal colon mucosa. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's t test.

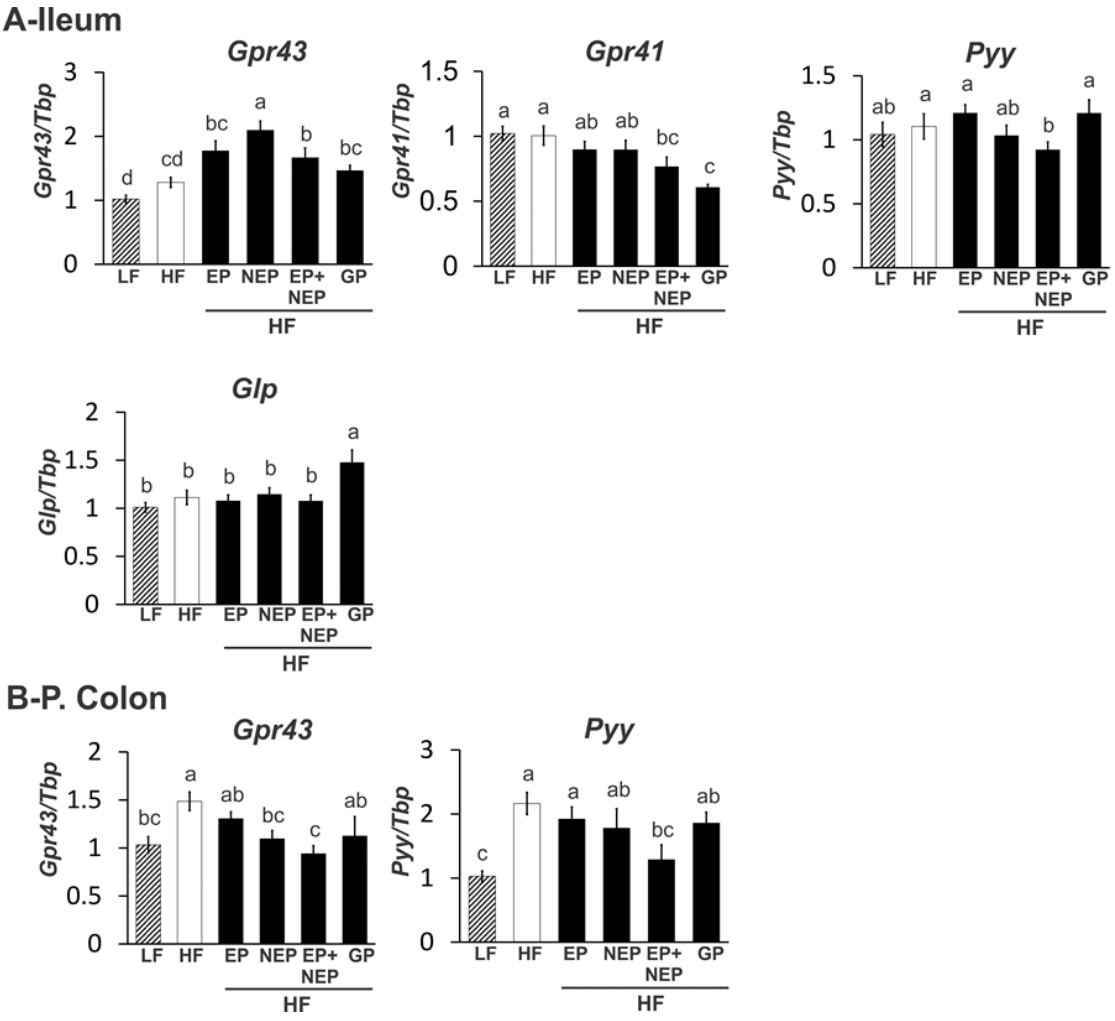

Figure 4.9. Expression of G-Protein Receptors and Glycoproteins in Intestinal Mucosa. The expression of G-protein receptors (*Gpr*) *41* and *43*, peptide YY (*Pyy*), and glucagon like protein (*Glp*) were measured as markers of SCFA regulation in the (A) ileum and (B) proximal colon mucosa of C57BL/6J mice fed a low fat (LF),high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. qPCR was conducted to measure mRNA abundance of genes associated with regulation of energy intake in ileum mucosa and proximal colon mucosa. Means ± SEM (n=9-10) without a common lowercase letter differ ($p<0.05$) using one-way ANOVA and Student's test.

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CHAPTER V

EPILOGUE

Summary of Findings

The prevalence of obesity has reached epidemic levels worldwide, and is associated with excess energy consumption and low levels of physical activity [1]. The numerous health consequences associated with obesity such as cardiovascular disease, hypertension, and type 2 diabetes are referred to as the metabolic syndrome [2]. Increased adiposity due to adipocyte hyperplasia or hypertrophy initiates inflammatory signaling that can result in impaired insulin signaling and other metabolic complications [3]. Because of the great prevalence of these conditions, identification of dietary strategies to prevent and treat obesity is warranted.

One potential way to combat chronic inflammation and health complications associated with diet-induced obesity is through consumption of polyphenol-rich table grapes. Grapes and grape products have been demonstrated to reduce oxidative stress, inflammation, and insulin resistance and improve heart health [4, 5, 6]. However, the mechanisms by which they exert these beneficial effects and the specific compounds in grapes responsible for mediating these effects remain in question. While grapes are rich in polyphenols, their bioavailability is poor, estimated to be less than 5%. Because of this, it is hypothesized that the polyphenols and fiber in grapes reach the cecum and colon

and impact gut microbial populations that in turn are responsible, in part, for the observed systemic health benefits. It has been demonstrated that grape products or several of their components increase beneficial microbial species including *Lactobacillus* spp. and *Enterococcus spp.* [7, 8]. Additionally, gut microbial fermentation of grape polyphenols and fiber may result in short chain fatty acid (SCFA) production, some of which may improve colonocyte health, reduce inflammation, and improve insulin sensitivity [9,10,11,12].

However, the bioavailability of grape polyphenols and the interactions with gut microbes are still in question, as is their influence on diet-induced obesity. Therefore, I investigated the following two specific aims;

Aim #1) Determine the extent to which California table grapes attenuate body fat accumulation, systemic inflammation, insulin resistance, and impact gut microbiota in mice fed an American type diet rich in butter (CHAPTER III); and

Aim #2) Identify the key bioactive fraction(s) responsible for reducing adiposity, inflammation, and insulin resistance, and modulating gut microbiota in mice fed an American type diet rich in four types of saturated fat (CHAPTER IV).

The results of the first study demonstrate that consuming one or both levels of table grapes (3-5%, w/w) attenuated the accumulation of body and liver fat in mice fed a diet rich in butter (i.e., 35% kcals from fat, most from butter; and amount of fat similar to the 50th percentile of American fat consumption) compared to control mice. However, these lipid-lowering effects were not associated reductions in markers of inflammation in white adipose tissue (WAT) or improvements in glucose tolerance. Interestingly, populations of

the deleterious sulfidogenic bacteria *Desulfobacter* spp, and the *Bilophila wadsworthia*-specific dissimilatory sulfite reductase gene were decreased by grape consumption and populations of the beneficial bacterium *Akkermansia muciniphilia* tended to be higher in grape-fed mice compared to their respective controls. These data indicate that grape consumption (3 or 5%, equivalent to 9–15 servings) is effective in attenuating adiposity and hepatic steatosis, and altering gut microbial composition in mice fed a butter-rich diet. In the second study, it was observed that in mice fed a high fat American type diet (i.e., 45% kcals from a mixture of lard, shortening, butter, and beef tallow, which was equivalent in fat amount and type to the 75th percentile of American fat consumption), the extractable polyphenol fraction (EP) alone or when combined with the non-extractable polyphenol fraction (EP+NEP) reduced adiposity, liver and plasma triglycerides, mRNA markers of inflammation within WAT, and improved insulin sensitivity compared to the high-fat fed controls. The EP fraction is abundant in polyphenols, primarily anthocyanins, which I hypothesize are responsible for the beneficial outcomes observed. However, the mechanisms by which the EP fraction exerts these effects on adiposity, insulin sensitivity, hepatic steatosis, and inflammation remain unclear.

One unexpected outcome observed in the second study was that the 5% grape diet fed adversely impacted some of the outcomes (i.e., decreased glucose disposal and increased adiposity and inflammatory markers). The differences in the effectiveness of grape powder diet between the first and second study may be due to several factors. First, the high fat diet in the first study consisted of 34% fat (of total kcals) primarily from butter, while in the high fat diet in the second study consisted of 45% fat (of total kcals) from a combination

of lard, shortening, butter, soybean oil, and beef tallow. These differences in the fat content and source may have contributed to the differences observed in the effectiveness of the grape powder diet. In the second study, while the grape powder diet (GP) contained the same amount of polyphenols that are in the grape fraction diets (i.e. HF-EP, HF-NEP, and HF-EP+NEP), we did not observe the health benefits with the grape powder diet that we did with the fraction diets. It has been demonstrated that certain fibers and proteins interact with polyphenols and can make them either more or less bioaccessable [13]. Therefore, it is possible that the acidified methanol extraction process used to obtain the EP and NEP fractions liberated the polyphenols from the fiber or other components (e.g., proteins) they are bound to, thereby making them more bioaccessable. However, further research is needed to determine if the extraction process indeed makes the polyphenols more bioaccessable, and thus more bioavailable. We did not attempt to measure the polyphenol profile in the plasma of mice consuming EP, NEP, or GP because we anticipated that the plasma levels would be undetectable due to our mice being fasted and the knowledge from a previous study indicated that polyphenol levels spike within the first 3 hours following ingestion [6]. One possible future study would be to gavage mice with EP, NEP, or GP and collect blood 1-3 hours post gavage to use for examination of polyphenol content. While this would help greatly in providing information on the bioaccesability of the polyphenols in GP and its fractions, it would require a great deal of resources due to the expense of instrumentation and number of polyphenol standards that would have to be purchased for analysis.

Future Data Collection

In order to answer some of the remaining questions in CHAPTER III and CHAPTER IV, additional analysis are warranted as follows:. (i) In CHAPTER IV, mesenteric WAT gene expression and lipid metabolism, (ii)microbial profile and SCFA composition in the cecum, (iii) immunohistochemistry of the tight junction protein ZO-1 in the ileum, and (iv) examination of protein levels of 5' adenosine monophosphate kinase (AMPK) and genes that were altered by grape and grape extract treatments.

Future Research

Based on the findings in CHAPTER III and CHAPTER IV, pursuit of the following research questions is warranted: (i) Which specific anthocyanins present in the EP fraction are the most effective in preventing the negative effects of diet-induced obesity?; (ii) Is the EP fraction and specific anthocyanins within it effective in reducing adiposity and insulin resistance in subjects that are already obese (i.e., can it be used as a treatment for obesity versus as a prevention in the current study)?, (iii) What metabolites of grape polyphenols are being produced by the gut microbiota and are they responsible for the reductions in inflammation, adiposity, and insulin resistance?, and (iv) What was the reason for the discrepancies between the studies with regards to the differential effects of grape powder on body fat, insulin sensitivity, and inflammatory markers?

Q1. Which specific anthocyanins *present in the EP fraction are the most effective at preventing the negative effects of diet-induced obesity?*

Our data showed that the EP fraction from grapes was more effective than the NEP or whole grape diet in preventing adiposity, insulin resistance, systemic inflammation, and hepatic steatosis in mice fed an American-type, high fat diet (i.e., 45% kcals from fat with a mixture of saturated fatty acids). The EP fraction contains many different anthocyanins in differing amounts and thus examination of their individual effectiveness is needed. To examine this, I propose an in vitro screening of individual anthocyanins for suppression of NF-kB activity, as conducted by Chuang et al. [14]. Primary cultures of human adipocytes will be transiently transfected with a NF-kB responsive luciferase reporter construct. Cells will be pretreated with vehicle or three different doses of malvidin, cyanidin, or peonidin for 1 h and then treated with or without 100 ng/mL TNFα for 24 hours. Measurement of firefly luciferase activity relative to control renilla luciferase activity will be used to determine the effect of the anthocyanins treatment on suppression of NF-kB activity. The outcomes of this experiment will demonstrate which anthocyanins present in grapes exhibits the greatest anti-inflammatory effects in primary adipocytes. Subsequently, the most effective candidate anthocyanins identified using this screening strategy would be included in the diets of high fat fed mice alone or in combinations and the outcomes measured in Chapter IV would be assessed.

Q2. Is the EP fraction and specific anthocyanins within it effective in reducing adiposity and insulin resistance in obese subjects?

The number of individuals who are overweight or obese and exhibit symptoms of the metabolic syndrome is at epidemic levels in the U.S. Our previous data showed that the EP fraction exerted the greatest impact on preventing diet-induced obesity and related complications in mice fed a high fat diet. Because this was a preventative model (i.e., mice were given the high fat diet at the onset of the study rather than after they gained body fat and became insulin resistant), it doesn't take into account the effect on individuals who are already obese. Therefore, a model of the effects of the EP fraction on reducing the effects of diet-induced obesity in mice that are already obese and exhibiting symptoms of the metabolic syndrome would be beneficial to provide data from a treatment perspective. To examine this question, I would employ a 16 week study using C57BL/6 mice fed a low fat (LF) or a high fat (HF) American type diet for 6 weeks to make them overweight and insulin resistant, as described by Shen et al [15]. Subsequently, mice would by randomization into various treatment groups for the remaining 10 weeks as follows: LF, HF, HF plus anthocyanin #1, HF plus anthocyanin #2, HF plus anthocyanin #1 and #2, and HF plus EP. The most effective anthocyanins identified through exploration of Q1 will be utilized as "anthocyanin #1" and "anthocyanin #2" in this study. At the end of the study, mice will be killed and WAT, liver, intestines, and plasma will be collected and used for analysis, as described in CHAPTER III and IV. The outcomes of this experiment will provide information on the effectiveness of grape anthocyanins on treatment of diet-

induced obesity as well as identify which grape anthocyanins are most effective at attenuating diet-induced obesity and associated complications *in vivo*.

Q3. What metabolites of grape polyphenols are being produced by the gut microbiota and are they responsible for the reductions in inflammation, adiposity, and insulin resistance?

Our data demonstrated that feeding grapes or their methanol-extractable fractions alters the gut microbial populations in mice fed a high fat diet. Research has illustrated that anthocyanins are not readily bioaccessable or bioavailable [16]. Because of this, it can be hypothesized that the gut microbiota are interacting with the grape anthocyanins to produce metabolites that may be responsible for reducing inflammation, adiposity, insulin resistance, or hepatic steatosis. It has been observed that metabolites of anthocyanins such as gallic acid and syringic acid are indeed bioavailable and possess anti-inflammatory and antioxidant properties [17]. To explore the metabolite production by gut microbiota in response to grape polyphenols, I propose an in vitro model. The specific beneficial bacterial strains identified to be altered upon anthocyanin feeding in Q2 will be cultured and exposed to candidate anthocyanins alone, or the EP fraction of grapes. Growth of the bacteria will be monitored over 24 hours and then the conditioned media will be collected and analyzed for metabolite composition. Subsequently, the conditioned media will be used for treatment of primary human adipocytes transfected with a NF-kB responsive luciferase reporter construct to examine its effect on inflammation, as discussed in detail in Q1. The outcomes of this experiment will identify the metabolites produced by

beneficial bacterial strains in response to grape anthocyanin exposure as well as the effectiveness of these metabolites on suppressing inflammation.

Q4. What was the reason behind the discrepancies between the two studies in regards to the differential effects of grape powder on body fat, insulin sensitivity, and inflammation?

In the first study (CHAPTER III), our data demonstrated that feeding grape powder at one or both levels (i.e., 3% or 5% w/w) in conjunction with a high fat diet (i.e.34% of kcal primarily from butter) reduced body fat, and reduced markers of inflammation in WAT. However, in the second study feeding grape powder at the 5% level in conjunction with a high fat diet (i.e. 45% of kcal from mixed sources) did not result in improvements in body fat, insulin sensitivity, and inflammation. This may be due to several different factors including; (i) the differences in fat content and composition, (ii) the length of the studies, and (iii) the diet formulation process.

With the first study, the fat content of the HF diets was 34% of total kcals primarily from butter, while in the second study the HF diets contained 45% of total kcals from mixed sources of fats, representative of the fat distribution of a typical American diet. These differences in fat content and sources may have contributed to the differences we observed in the effectiveness of the grape diets between the first study and the second study. It is possible that the inflammatory and lipogenic response was much greater in the 45% kcal from mixed fats diet and that the effect of grape feeding was not able to overcome this increased response like it did in the 34% kcal from butter fat fed mice.

The differences in length of the two studies may also explain some of the data we observed in the GP group. The duration of the first study was 11 weeks and we did observe improvements in adiposity and markers of inflammation with the 5% grape fed mice while the second study was 16 weeks and we did not observe any improvements in inflammation or adiposity in the mice fed the same amounts of powdered grapes. During the second study, we did harvest three animals per treatment at week 12 and found that the gene expression of inflammatory markers (i.e. *Mcp1, F4/80, Cd11c*) in WAT was not as robust in the GP group as what we observed in animals harvested at week 16. This may indicate that the powdered grape diet has a more acute rather than chronic effect on inflammation. Consistent with this finding, it was previously shown that GP supplementation (i.e 3% w/w) to a HF diet (i.e 60% of total kcals from fat) results in improvements in glucose tolerance at week 5 but not at weeks 10 and 15 [6].

Additionally, because we did not make the diets ourselves, it is possible that during the diet formulation process the mild amounts of heat and mixing used during pelting of the diet may have introduced variation or altered the compounds present in our GP diets. We did attempt to have the diets analyzed for phenolic content after they were made, but were unsuccessful. This may be due to the amount of fat in the diets that may interfere with the ability to isolate and detect phenolic compounds. To eliminate this potential confounding variable, in future studies using daily oral gavage of the EP, NEP, and GP components would ensure that these compounds are delivered to each mouse in the same dosage and also eliminate the variation that may be introduced during the diet formulation process.

Conclusion

In summary, there is still much to be elucidated concerning the bioaccesability, bioavailability, and metabolic actions of grape polyphenols on attenuation of diet-induced obesity, chronic inflammation, insulin resistance, and hepatic steatosis. Additionally, it is necessary to understand the way in which these polyphenols interact with gut microbiota to produce metabolites that may be more bioavailable and thus responsible for the prevention of adiposity, insulin resistance, and inflammation associated with consumption of a high fat diet. The proposed studies will help in answering these questions which will allow for the development of dietary means by which to use grapes or their components to prevent and or treat diet-induced obesity and incidence of metabolic syndrome.

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